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# Conversion of tumor-specific CD4+ T-cell tolerance to T-cell priming through *in vivo* ligation of CD40

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**Tumor antigen-specific T-cell tolerance limits the efficacy of therapeutic cancer vaccines. Antigen-presenting cells mediate the induction of T-cell tolerance to self-antigens. We therefore assessed the fate of tumor-specific CD4+ T cells in tumor-bearing recipients after *in vivo* activation of antigen-presenting cells with antibodies against CD40. Such treatment not only preserved the responsiveness of this population, but resulted in their endogenous activation. Established tumors regressed in vaccinated mice treated with antibody against CD40 at a time when no response was achieved with vaccination alone. These results indicate that modulation of antigen-presenting cells may be a useful strategy for enhancing responsiveness to immunization.**

The foundation of cancer immunotherapy rests on the ability of the adaptive immune response to specifically recognize and reject cancer cells in the tumor-bearing host. Although multiple effector mechanisms can be recruited to participate in tumor rejection, it is the T-cell arm of the response that achieves tumor specificity, and CD4+ T cells in particular that orchestrate the activities of both antigen-specific as well as nonspecific elements of the tumoricidal response[1-5].

In a T-cell receptor (TCR) transgenic model, CD4+ T cells specific for a model tumor antigen are rendered tolerant early in the course of tumor progression[6]. This tolerance is antigen-specific, and occurs when other elements of the T-cell repertoire function normally. Given the central role of CD4+ T cells in the anti-tumor immune response, defining the mechanism(s) responsible for the induction of CD4+ T-cell tolerance to tumor antigens is necessary for the successful development of therapeutic cancer vaccines[7].

Studies of T-cell tolerance to peripheral self-antigens have demonstrated that bone marrow-derived antigen-presenting cells (APCs) are involved in the induction of tolerance to antigens expressed by non-hematopoietic tissues[8]. Given that host APCs are also required for initiating productive T-cell responses, the state of activation and/or differentiation of the APC may be the requisite determinant of whether T cells are primed or rendered tolerant.

The engagement of CD40 on APCs by its ligand CD154 on CD4+ T cells is an important event during APC activation[9,10]. CD40 ligation on dendritic cells results in the upregulation of costimulatory molecules and the secretion of inflammatory cytokines that are central to the initiation of cell-mediated immune responses. We therefore sought to determine whether the *in vivo* activation of APCs using CD40-activating antibodies could provide the signal(s) needed to induce activation rather than tolerance of CD4+ T cells. Using a lung metastasis model of a murine renal-cell carcinoma, we found that CD40 ligation not only preserved the responsiveness of tumor-specific CD4+ T cells to immunization, but also led to their endogenous activation in the absence of vaccination. Furthermore, immunization of tumor-bearing mice previously treated with antibody against CD40 resulted in substantial tumor rejection, which was not found after vaccination alone. These results indicate that modulating host responsiveness to immunization through APC activation may be a useful strategy to enhance the efficacy of tumor vaccines.

## CD40 ligation preserves responsiveness to vaccination

CD4+ T cells specific for an antigen expressed exclusively by a B-cell lymphoma are rendered tolerant during the course of tumor progression[6]. Similar findings have been obtained in a lung metastasis model of renal cell carcinoma (E.M.S. *et al.*, manuscript in preparation). In both systems, tumor antigen-specific transgenic CD4+ T cells transferred into a tumor-bearing host undergo a transient clonal expansion and have a phenotype associated with antigen recognition. However, functional analysis demonstrates that these cells have a diminished response to peptide antigen *in vitro*, and are unable to be primed *in vivo*.

We determined whether *in vivo* ligation of CD40 could prevent the development of unresponsiveness of TCR transgenic CD4+ T cells specific for an MHC class II epitope of influenza hemagglutinin[11] (HA) after their transfer into mice with established pulmonary metastases of a renal cell carcinoma expressing HA (RencaHA). Immunization of non tumor-bearing mice with a recombinant vaccinia encoding-HA (vac-HA) resulted in a clonal expansion of HA-specific T cells (Fig. 1). The percentage of clonotype-positive T cells in the spleen of a vac-HA primed mouse 6 days after immunization was almost 400% greater than the frequency in an unimmunized mouse (1.42% and 0.37%, respectively; Fig. 1*a*). However, the response to vac-HA immunization was substantially impaired in a RencaHA-bearing mouse, resulting in a minimal increase in the percentage of clonotype-positive T cells relative to an unimmunized tumor-bearing mouse (0.71% and 0.58%, respectively). In contrast, treatment of tumor bearing

**Table 1** Effect of xenogeneic immunoglobulin on T cell responsiveness

| Group | IL-2 Mean pg/ml per 100 clonotype $^+$T cells | IFN-γ Mean pg/ml per 100 clonotype $^+$T cells |
|---|---|---|
| No tumor | 5.9 ± 2.3 | 137 ± 80 |
| No tumor + rat IgG control | 8 ± 3.2 | 163 ± 47 |
| Renca HA | 1.9 ± 0.2* | 41 ± 3.0* |
| Renca HA + rat IgG control | 2.4 ±0.6* | 54 ± 6.0* |
| Renca HA + anti-CD40 | 7.4 ± 0.4 | 197 ± 5.0 |

RencaHA-bearing mice or tumor-free mice received anti-HA TCR+ transgenic T cells and were treated with either the agonist CD40 antibody FGK45 or a similar amount of polyclonal rat IgG control antibody. On day +15 after T cell-transfer, all the mice were immunized with vac-HA and were killed 6 d later; production of IL-2 and IFN-γ was determined by ELISA. Data represent mean + s.e.m. of triplicate cultures from three mice in each group, and are expressed as the amount of cytokine produced per 100 clonotype-positive T cells. *Not statistically significant.

mice with activating antibody against CD40 (FGK45, ref. 12) resulted in preservation of the response to vac-HA, as demonstrated by a clonal expansion that was similar to that in a tumor-free mouse (1.47% versus 1.42%, respectively). Statistical comparison of the response to immunization (Fig. 1*b*) verified that tumor-bearing mice were significantly impaired in their response to vac-HA priming compared with tumor-free mice ($P$ = 0.003). Furthermore, this response was preserved in RencaHA-bearing mice treated with activating antibodies against CD40 ($P$ = 0.007).

### CD40 ligation results in preservation of CD4$^+$ T-cell function

T cells from vaccinated tumor-free mice fulfill several functional criteria indicative of effective T-cell priming—*in vivo* clonal expansion (Fig. 1*b*, stippled bar), increased IL-2 production (Fig. 2*a*, stippled bar) and differentiation into effector cells capable of producing gamma interferon (IFN-γ) after *in vitro*-stimulation with HA peptide (Fig. 2*b*, stippled bar). Most of the increase in the antigen-specific proliferative response after immunization is attributable to the increased numbers of antigen-specific T cells that are generated[13], such that on a 'per-cell' basis, HA-specific proliferation does not reflect priming (Fig. 2*c*).

Analysis of HA-specific cytokine release from the splenocytes of RencaHA-bearing mice showed significant impairment in their capacity to produce IL-2 ($P$ = 0.005) and IFN-γ ($P$ < 0.001; Fig. 2 *a* and *b*). This unresponsiveness was antigen-specific, as demonstrated by the equivalent responses to vaccinia antigens *in vitro* of T cells obtained from these same vac-HA primed, RencaHA-bearing mice and non-tumor-bearing mice (data not shown). In contrast, treatment of RencaHA-bearing mice with antibody against CD40 preserved the IL-2 ($P$ < 0.001) and IFN-γ ($P$ < 0.001) response to peptide antigen. Although T cells from tumor-bearing mice treated with antibody against CD40 proliferated more after HA peptide treatment *in vitro* than those from untreated mice, this difference was not statistically significant (Fig. 2*c*). Overall, the *in vivo* ligation of CD40 in mice with metastatic RencaHA resulted in a degree of clonal expansion and production of IL-2 and IFN-γ that was similar to that in immunized mice without tumors.

To insure that the observed effects of antibody treatment were a consequence of CD40 ligation rather than a result of the host response to xenogeneic protein (FGK45 is a rat IgG), we compared the effect of antibody against CD40 with that of a control antibody (polyclonal rat IgG) after injection into tumor-bearing mice (Table 1). Whereas treatment of tumor-bearing mice with antibody against CD40 preserved the ability of clonotype-positive T cells to produce IL-2 and IFN-γ in response to vac-HA (similar to immunized tumor-free mice), T cells from tumor-bearing mice treated with control antibody (and mice not receiving antibody) remained impaired in these responses.

### Endogenous priming of tumor-specific T cells by CD40 ligation

If ligation of CD40 leads to the activation of APCs that have processed tumor antigen, one might expect this to result in the priming of tumor-specific T cells, even in the absence of immunization. To address this, we assessed the function of clonotype-positive CD4$^+$ T cells isolated from the draining lymph nodes (peritracheal, peribronchial and mediastinal) of mice with pulmonary metastases of RencaHA. As in other tumor systems,

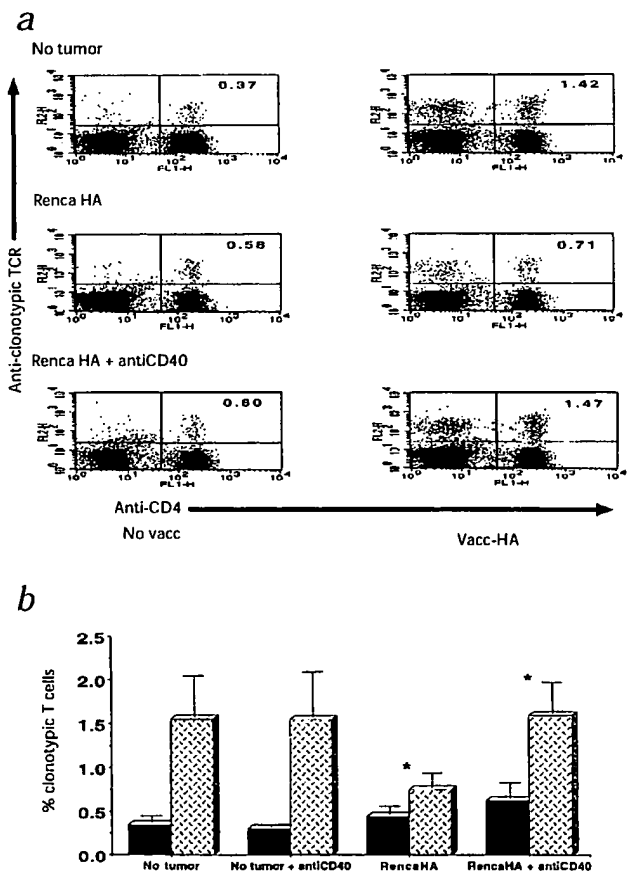



**Fig. 1** Effect of CD40 ligation on the responsiveness of RencaHA bearing mice to vaccination. Tumor-bearing mice or tumor-free mice received anti-HA TCR$^+$ transgenic T cells and were treated with the agonist CD40 antibody FGK45 (antiCD40). Mice were immunized with vacc-HA 15 d after T-cell transfer and were killed for analysis 6 d later. T cells were analyzed by two-color flow cytometry staining for CD4 versus anti-HA TCR clonotype. ***a***, Two-color FACS analysis of splenocytes from non-tumor-bearing and RencaHA-bearing mice. T cells from unimmunized mice (No vac) or immunized mice (vac-HA) were analyzed. Upper right quadrant numbers, percentage of double-positive T cells. ***b***, T cells from unimmunized mice (■) and vac-HA-immunized mice (▨) were analyzed by flow cytometry. Data represent mean + s.e.m. of the percentage of T cells co-expressing CD4 and the clonotypic TCR for six mice/group (combined results of two independent experiments with three mice per group per experiment). *, $P$ = 0.007 for the difference in clonal expansion in response to vac-HA between RencaHA and RencaHA/anti-CD40.

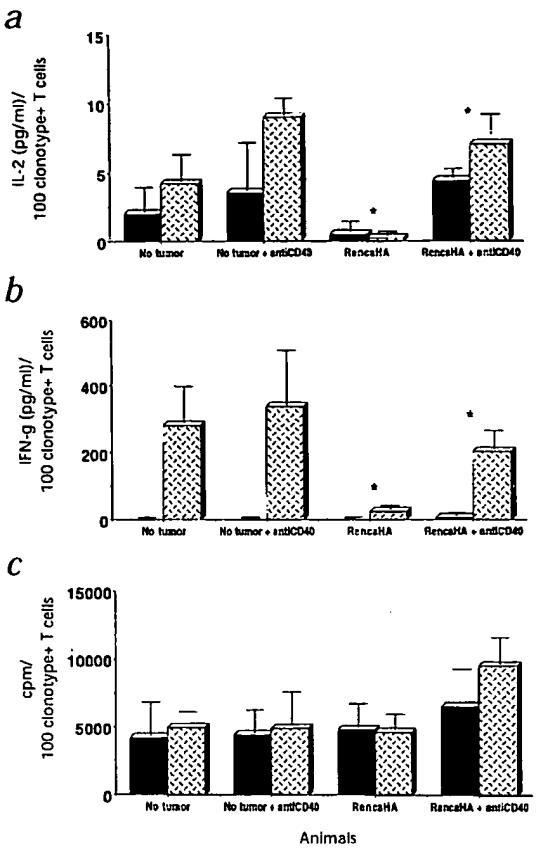



**Fig. 2** Effect of *in vivo* ligation of CD40 on the functional responses of antigen-specific CD4⁺T cells. T cells from the mice in Fig. 1 were isolated and assessed for their functional response to HA peptide *in vitro*. ***a*** and ***b***, Production of IL-2 (*a*) and IFN-γ (*b*) in response to *in vitro* stimulation with $HA_{110-120}$ peptide. Purified T cells from unimmunized (■) or vac-HA immunized (⊠) mice were stimulated with HA peptide for 48 h; supernatants were collected and assayed for IL-2 or IFN-γ by ELISA. Data represent mean + s.e.m. of triplicate cultures from six mice in each group, and are expressed as the amount of cytokine produced per 100 clonotype-positive T cells. *, $P < 0.001$. ***c***, Proliferative response to stimulation with HA peptide. T cells from unimmunized (■) or vac-HA immunized (⊠) mice were mixed with fresh splenocytes and HA peptide. Data represent mean +s.e.m. of the cpm per 100 clonotype-positive T cells per well.

there was an expansion of the population of clonotype-positive T cells in RencaHA-bearing mice relative to that in tumor-free mice (Fig. 3*a*). This expansion was enhanced in RencaHA-bearing mice treated with activating antibodies against CD40, although this difference was not statistically significant. Similarly, there was a trend towards increased proliferation in response to HA peptide *in vitro* in T cells isolated from tumor-bearing mice treated with antibody against CD40 (Fig. 3*b*). However, an analysis of cytokine release in response to HA peptide showed that clonotype-positive T cells from tumor-bearing mice treated with antibody against CD40 secreted more IL-2 than those from either naive or untreated tumor-bearing mice ($P = 0.036$; Fig. 3*c*). Furthermore, these cells had acquired the capacity to produce IFN-γ even in the absence of *in vivo* priming with vac-HA ($P = 0.01$; Fig. 3*d*). Whereas the IFN-γ production on a 'per-cell' basis was significantly lower than that in response to vac-HA priming (18 and 200 pg/ml per 100 clonotype-positive cells, respectively), the capacity of tumor-specific T cells to produce this cytokine is indicative of endogenous differentiation into functional effector cells.

### Anti-tumor effect of treatment with antibody against CD40

Given that CD40 ligation preserved the responsiveness of RencaHA-bearing mice to vac-HA priming, we determined whether treatment with antibody against CD40 alone or in combination with vaccination had any anti-tumor effect in this pulmonary metastasis model of renal cell carcinoma. The lung of an untreated RencaHA-bearing mouse contained many malignant nodules 3 weeks after T-cell transfer (Fig. 4). Vac-HA given 6 days before the mouse was killed did not have any demonstrable anti-tumor effect (Fig. 4, Vac-HA), as expected, given the T-cell unresponsiveness demonstrated above. Further analysis of six mice per group demonstrated that all unimmunized or vac-HA-immunized mice examined at this time had more than 20 pulmonary metastases (data not shown). Similarly, the lungs of mice receiving control (polyclonal rat IgG) antibody, either alone or in combination with vac-HA, had a tumor burden that was indistinguishable from that of untreated mice.

In a group of mice treated with antibody against CD40 alone (given on days −1 and +1), four of the six mice had more than 20 large tumor nodules at the time of analysis. One mouse that was initially thought not to have tumor by macroscopic examination of the lung was subsequently found to have malignant nodules by histologic examination (Fig. 4, Anti-CD40). The other mouse was tumor-free, as ascertained by careful histologic examination. This modest anti-tumor effect of treatment with antibody against CD40 alone may be a consequence of the endogenous activation of T cells (Fig. 3).

Whereas vac-HA immunization given 15 days after T-cell transfer led to no substantial anti-tumor response, identical immunization of RencaHA-bearing mice that were previously treated with CD40-activating antibodies resulted in a definite anti-tumor effect. Of the six mice treated in this way, two had neither macroscopic nor microscopic evidence of pulmonary metastases at the time of analysis (day +21). Furthermore, *in vitro* culture of lung explants from these mice failed to show any tumor growth (data not shown). The remaining four mice had fewer than ten pulmonary nodules per mouse (a tumor burden equivalent to that in untreated mice 1 week after the transfer of T cells), indicative of a substantial delay in tumor growth. A representative photograph of the lung of one of these mice is shown in Fig. 4 (Anti-CD40/Vac-HA). The modest cellular infiltrate in the pulmonary

**Table 2** Comparison of IL-4 and IFN-γ responses in tumor-bearing mice and peptide-treated mice.

| Group | IL-4 Mean pg/ml per 100 clonotype⁺ T cells | IFN-γ Mean pg/ml per 100 clonotype⁺ T cells |
|---|---|---|
| Renca HA | 0 | 24 ± 5 |
| Renca HA + anti-CD40 | 0.15 ± 0.15 | 115 ± 5 |
| HA peptide | 0.85 ± 0.05* | 32 ± 1 |
| HA peptide + Anti-CD40 | 2.3 ±0.8* | 40 ± 9* |

Tumor-bearing mice or tumor-free mice received anti-HA TCR⁺ transgenic T cells and were treated with the agonist CD40 antibody FGK45. Then, 2 d after T-cell transfer, the tumor-free mice were given a tolerogenic dose of HA peptide (275 μg). All the mice were immunized with vac-HA on day +9 after T-cell transfer and were killed 6 d later. Purified T cells were stimulated with HA peptide *in vitro* for 48 h and then supernatants were assayed for IL-4 and IFN-γ by ELISA. Data represent mean + s.e.m. of triplicate cultures from three mice in each group, and are expressed as the amount of cytokine produced per 100 clonotype-positive T cells.*$P = 0.006$, compared with RencaHA.

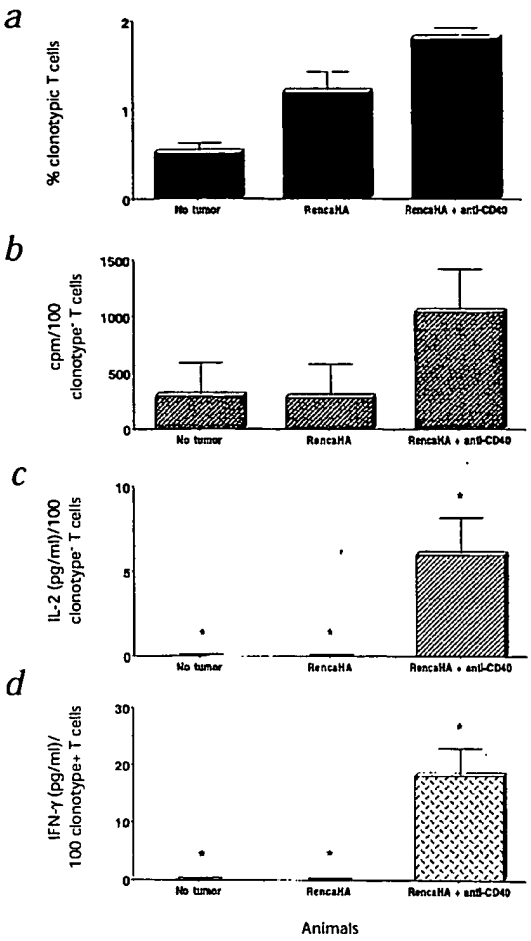
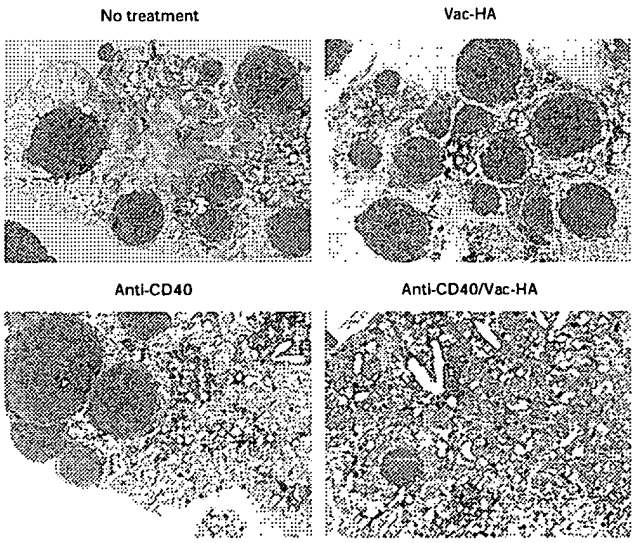



**Fig. 3** Analysis of HA-specific T cells isolated from regional lymph nodes. RencaHA-bearing mice or tumor-free mice received anti-HA TCR$^+$ transgenic T cells and were treated with antibody against CD40. On day +21, mice were killed and the thoracic lymph nodes from three mice per group were collected and pooled for analysis. ***a***, Lymph node cells were analyzed by two-color flow cytometry staining for CD4 versus anti-HA TCR clonotype. Data represent mean + s.e.m. of the percentage of double-positive T cells from two independent experiments. ***b***, Proliferative response to HA peptide. Data represent the mean + s.e.m. cpm per 100 clonotype-positive T cells per well from two independent experiments. ***c*** and ***d***, Purified T cells were stimulated with HA peptide for 48 h; supernatants were collected and assayed for IL-2 (*c*) or IFN-γ (d) by ELISA. Data represent mean + s.e.m. of triplicate cultures from three mice in each group, and are expressed as the amount of cytokine produced per 100 clonotype-positive T cells. *, $P = 0.036$, IL-2; *, $P = 0.01$, IFN-γ.

parenchyma of mice receiving antibody against CD40 and vac-HA was somewhat greater than that in the other groups, although the importance of this is uncertain.

### CD40 ligation prevents peptide induced CD4$^+$ T-cell anergy

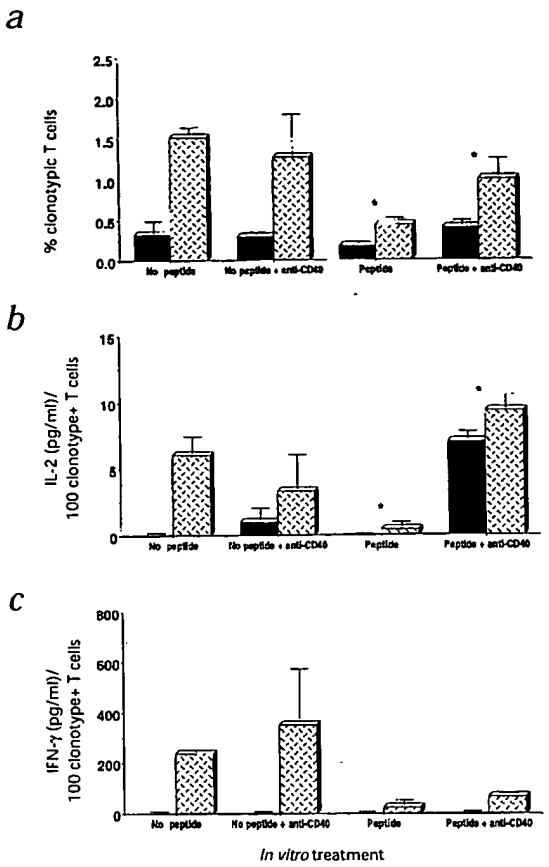
As the Renca tumor used in our system, like many tumors of epithelial origin[14-20], expresses CD40 and could thereby be recognized directly by FGK45, we sought to determine whether antibody against CD40 could prevent T-cell unresponsiveness in another well-characterized *in vivo* model of T-cell anergy: peptide-induced T-cell anergy[21,22]. Intravenous administration of HA peptide induced T-cell unresponsiveness to vac-HA (Fig. 5). Compared with those from mice not given peptide, clonotype-positive T-cell populations from mice receiving peptide failed to expand *in vivo* ($P < 0.001$), and were defective in their ability to produce IL-2 ($P = 0.001$) and IFN-γ ($P < 0.001$). In contrast, treatment with antibody against CD40 preserved the response to vac-HA in mice that received intravenous HA peptide. The clonal expansion of HA-specific transgenic T cells was significantly higher in peptide-treated mice given antibody against CD40 than in those receiving peptide alone ($P = 0.015$). Furthermore, treatment with antibody against CD40 prevented the loss of IL-2 production by clonotype-positive T cells in peptide-treated mice ($P < 0.001$). Treatment with antibody against CD40 alone seemed to enhance IL-2 production by T cells encountering an otherwise tolerogenic form of antigen *in vivo*, even in the absence of immunization with vac-HA.




In contrast to what was seen with tumor-induced T-cell tolerance, CD40 ligation in mice receiving HA peptide failed to preserve the ability of HA-specific CD4$^+$ T cells to differentiate into IFN-γ-producing effector cells in response to vac-HA priming (Figs. 2*b* and 5*c*). Given that these cells were responsive by other parameters (such as clonal expansion and IL-2 production), we looked for evidence of differentiation along another effector pathway: that is, the production of IL-4. Indeed, the peptide-induced tolerance model (but not the tumor tolerance model) seems to favor the differentiation of clonotype-positive T cells into IL-4-producing cells (Table 2). After treatment with antibody against CD40, the effector response to vac-HA priming is manifest as enhanced IL-4 production. Therefore, CD40 ligation preserves T-cell responsiveness in the peptide tolerance model, but the response is polarized towards the production of a prototypic Th-2 cytokine, which probably accounts for the failure of these cells to make IFN-γ.

**Fig. 4** Effect of CD40 ligation on the anti-tumor response to vaccination. Tumor-bearing mice received anti-HA TCR$^+$ transgenic T cells and were treated with the agonist CD40 antibody FGK45. Mice were immunized with vac-HA on day +15 after the adoptive transfer of clonotypic T cells and were killed for analysis 6 d later. Lung samples are from an untreated tumor-bearing mouse (No Treatment), a mouse immunized with vac-HA alone (vac-HA), a mouse treated with CD40 alone (Anti CD40) and a mouse treated with combination therapy (Anti CD40/Vac-HA).

Fig. 5 Effect of CD40 ligation on the response to intravenous HA peptide. Mice that received anti-HA TCR$^+$ transgenic T cells either were or were not treated with antibody against CD40 (FGK45) before receiving a tolerogenic dose of HA peptide (275 μg). Vac-HA was given 15 d after T-cell transfers, and analysis was done 6 d later. *a*, T cells isolated from unimmunized (■) and vac-HA immunized mice (⊠) analyzed by two-color flow cytometry staining for CD4 versus anti-HA TCR clonotype. Data represent the mean + s.e.m. of the percentage of T cells co-expressing CD4 and the clonotypic TCR for three mice/group. *, $P = 0.015$. *b* and *c*, Purified T cells were stimulated with HA peptide for 48 h, then supernatants were collected and assayed for IL-2 (*b*) or IFN-γ (*c*) by ELISA. Data represent mean + s.e.m. of triplicate cultures from three to four mice in each group. Data are expressed as the amount of cytokine produced per 100 clonotype-positive T cells per well. *, $P < .001$, IL-2.




## Discussion

Much attention has been given to the observation that tumor cells, typically being the transformed counterparts of 'non-professional' antigen-presenting cells, lack the capacity to express T-cell co-stimulatory molecules. In the absence of adequate co-stimulation, the direct encounter of T cells with tumor cells has been proposed as the basis for the development of tumor antigen-specific T-cell tolerance. However, the requirement for a direct T cell–tumor interaction in the development of tumor-specific CD4$^+$ T-cell tolerance is problematic. Most tumors of nonhematopoietic origin do not express MHC class II molecules (although expression can often be induced in the presence of IFN-γ and tumor necrosis factor (TNF-α). Moreover, naive T cells circulate mainly between the blood and the secondary lymphoid compartments, only entering the extra-lymphoid tissue spaces after activation and acquisition of effector function. Although solid tumors do often metastasize through the lymphatics (an event that may have important immunologic consequences), in our model of CD4$^+$ T-cell tolerance to renal cell carcinoma, we have not identified lymphatic tumor, indicating that tolerance does not depend on this event.

Alternatively, the induction of tumor-specific CD4$^+$ tolerance may involve the presentation of tumor antigen by host APCs. APCs are indispensable in establishing peripheral T-cell tolerance to normal self-antigens. Bone marrow-derived APCs can capture and present peripheral tissue-specific antigens to naive CD8$^+$ T cells, leading to their deletion[23,24]. There is a similar requirement for the processing of parenchymal self antigen by host APCs in the induction of CD4$^+$ T-cell tolerance[25].

In the immune response to tumors, one plausible scenario therefore involves the capture of tumor antigen at the tumor site by host APCs that then migrate to the T-cell zone of secondary lymphoid organs for presentation to tumor-specific T cells. Consistent with this is the observation that anti-HA CD4$^+$ T cells isolated from the draining lymph nodes and spleen of RencaHA-bearing mice undergo an initial clonal expansion accompanied by an increase in size (forward light scatter) and loss of naive phenotype, compared with that of HA-specific T cells from tumor-free mice and mice with Renca wild-type tumors (data not shown). These changes are consistent with transgenic T cells encountering antigen on cells capable of providing some degree of co-stimulation. Despite these changes, these same T cells are functionally impaired, as shown by their inability to proliferate *in vivo* in response to vaccination, as well as their decreased capacity to produce cytokines when re-stimulated with the nominal peptide antigen *in vitro* (Figs. 1*b* and 2).

Given that bone marrow-derived APCs are also essential for T-cell priming, these observations lead to the hypothesis that the differentiation and/or activation state of the APC is the central determinant of T-cell priming versus tolerance[26]. In their immature state, APCs such as dendritic cells have relatively low levels of MHC, co-stimulatory molecules and other adhesion molecules that participate in T-cell priming[27]. Nonetheless, immature dendritic cells can efficiently capture fragments from apoptotic cells[28] and present peptide antigens derived from this material in the T-cell zones of lymphoid tissues[29]. In the absence of APC acti-

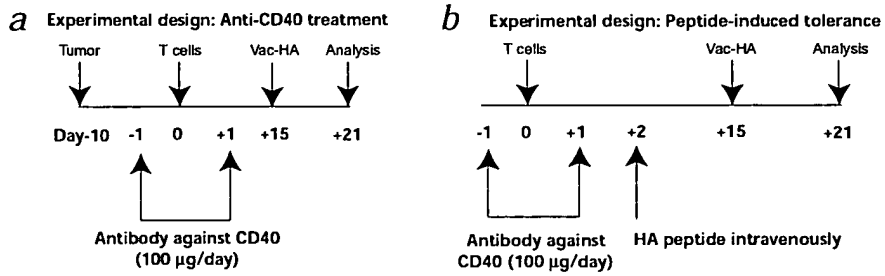



Fig. 6 Experimental protocols. Treatment and analysis protocols used for experiments in Figs. 1 and 2 (*a*) and Fig. 5 (*b*).

vation, this process has been proposed to mediate the induction and maintenance of peripheral tolerance to self antigens[8]. This pathway may well typify how the immune system normally encounters tumor antigens[30].

In contrast, the ability of the innate immune response to promote T-cell priming and cell-mediated immunity has been attributed to the production of factors that induce APCs to upregulate the expression of T-cell co-stimulatory molecules and to produce inflammatory cytokines[31-33]. This forms the basis of the efficacy of adjuvants often used in vaccine formulations. Therefore, strategies aimed at providing signal(s) that lead to effective APC activation *in vivo* have the potential to convert a T-cell encounter with antigen/APC from a tolerizing event into a priming event. Indeed, as demonstrated here, *in vivo* activation of APCs achieved through triggering of CD40 not only preserved the responsiveness of tumor-specific CD4$^+$ T cells to vaccination in tumor-bearing mice (Fig. 2) but also resulted in their endogenous activation rather than tolerance (Fig. 3). Similarly, *in vivo* ligation of CD40 on APCs resulted in the priming of CD4$^+$ T cells in response to an otherwise tolerogenic dose of peptide injected intravenously (Fig. 5).

The importance of APC activation through CD40 engagement has been recently emphasized by the demonstration that a prinicpal component of the T-helper cell function that is required for priming MHC class I-restricted CTLs is mediated through the engagement of CD40 on APCs by its ligand on CD4$^+$ T cells. The resulting activation of APCs is sufficient to drive naive CD8$^+$ T cells to become fully activated effector cells[34-36].

However, a model in which CD4$^+$ T cells alone are sufficient to activate APCs does not account for how CD4$^+$ T-cell responses can possibly be regulated. Specifically, if all that is required for the 'licensing' of APCs to activate T cells (including CD4$^+$ T cells themselves) is the cognate interaction between an antigen-specific T-helper cell and an APC presenting its antigen, then the outcome of all such encounters would be priming, including that of self-reactive T cells. Although there is definite evidence that the provision of T-helper cell function can indeed convert MHC class I-restricted CTL tolerance to T-cell priming[37,38], there is equally compelling evidence that CD4$^+$ T cells can be rendered tolerant *in vivo*[6,22,25,39-43], as was seen here.

Perhaps after an initial encounter with antigen, naive CD4$^+$ T cells are often not capable of providing sufficient signals to activate or 'license' an otherwise immature APC. The outcome of such an encounter would be CD4$^+$ T-cell tolerance. The fact that CD4$^+$ T-cell tolerance is seen even at the experimentally high CD4$^+$ T-cell precursor frequencies used in this and other studies further emphasizes the point that the availability of antigen-specific T helper-cell action alone cannot be the sole determinant regulating the induction of cell-mediated immunity.

Instead, it is likely that the pathway leading to APC activation is normally initiated as a consequence of the innate immune response to a pathogen and/or as a direct response of APCs to infection[34]. One of the most salient changes in the phenotype of activated versus immature dendritic cells is the increased expression of CD40 (ref. 27), as occurs after exposure to bacterial lipopolysaccaride[44]. Perhaps this renders the APC more receptive to T-helper cell function. Given the profound impairment of the ability to prime T-cell responses in mice with the targeted disruption of CD40 or CD40 ligand, it is likely that CD4$^+$ T cells are important in sustaining and perhaps amplifying APC activation after it is initiated. Anergic CD4$^+$ T-cells are very deficient in their ability to upregulate CD40 ligand[45]. We are now assessing the ability of HA-specific CD4$^+$ T cells from RencaHA-bearing mice to express CD40 ligand.

From a therapeutic perspective, it seems that this 'insufficient cross-talk' between tumor-specific CD4$^+$ T cells and host APCs in tumor-bearing mice is either corrected (if defective) or at least augmented after the exogenous triggering of CD40 with antibodies. The activation of APCs using this strategy not only converted T-cell tolerance to T-cell activation, but also preserved the responsiveness of tumor bearing mice to vaccination. Therefore, the advances made in vaccination against infectious pathogens using CD40-activating antibodies as 'adjuvants' (refs. 46,47) has now been extended to the field of tumor vaccines, indicating that modulation of APCs may be useful in enhancing the efficacy of this therapeutic modality.

## Methods

**Mice.** Male BALB/c mice 6–8 weeks old were obtained from the National Institutes of Health (Frederick, Maryland). TCR transgenic mice expressing an αβ T-cell receptor specific for amino acids 110–120 from influenza hemagglutinin presented by I-E$^d$ were a gift from H. von Boehmer[11]. These mice were crossed to a BALB/c background for more than ten generations. The transgenic mice used in these experiments were heterozygous for the transgene. All experiments involving the use of mice were in accordance with protocols approved by the Animal Care and Use Committee of the Johns Hopkins University School of Medicine

**Tumor cells.** Renal cell carcinoma cells (Renca) were obtained from the American Type Culture Collection (ATCC, Rockville, Maryland). Cells were cultured *in vitro* in RPMI 1640 media, supplemented with 10% FCS, 50 U/ml penicillin/streptomycin, 2 mM L-glutamine, and 50 mM β-mercaptoethanol (complete media), and were grown as an adherent population at 37 °C, 5% $CO_2$. RencaHA was generated by calcium phosphate-mediated plasmid transfection with the construct pIHA, which encodes the HA molecule of the influenza virus A/PR/8/34 (H1N1), as reported[48]. RencaHAneo was selected and grown in complete media supplemented with the neomycin analog G418 (400 μg/ml).

**Adoptive transfer.** Single-cell suspensions were made from peripheral lymph nodes and spleen collected from TCR transgenic donors. The percentage of lymphocytes double-positive for CD4 and the clonotypic TCR was determined by flow cytometry. Cells were washed three times in sterile Hanks balanced salt solution (HBSS), and injected into the tail veins of male BALB/c recipients such that a total of $2.5 \times 10^6$ CD4$^+$ anti-HA TCR$^+$ T cells was transferred to each recipient. RencaHA cells used for *in vivo* tumor challenge were detached from the culture flasks with trypsin (Sigma) and were suspended in complete media. Then, cells were counted and viability was assessed by trypan blue exclusion. If the viability was 100%, tumor cells were washed three times in sterile HBSS, and injected through tail vein in a total volume of 0.2 ml, $1 \times 10^5$ tumor cells per mouse.

***In vivo* treatment with activating antibodies against CD40.** The experimental design in Fig. 6a was used in the experiments in Figs. 1 and 2. Pulmonary metastases of RencaHA were established in BALB/c mice by intravenous injection of $1 \times 10^5$ tumor cells. After 10 d, transgenic anti-HA CD4$^+$ T cells ($2.5 \times 10^6$) were transferred intravenously into these recipients or into tumor-free mice (day 0). Half the mice in each group received 100 μg/day of the agonist CD40 antibody FGK45 given intravenously on days −1 and +1. Similarly, a subgroup of mice received 100 μg of polyclonal rat IgG (Sigma) intravenously on days −1 and +1 after T-cell transfer. On day +15 after T-cell transfer, half the mice in each subgroup were immunized subcutaneously with $1 \times 10^7$ plaque-forming units of a recombinant vaccinia virus encoding influenza hemaglutinin (vac-HA). In all experiments, three mice per subgroup were used and mice were analyzed individually. Each mouse was given a unique identification number so that specific determinants of T-cell responsiveness could be correlated within an individual as well as between mice in the same group or between groups. Mice were killed 6 d after immunization (day +21 after T-cell transfer) for analysis.

**Assessment of pulmonary metastases.** Mice were killed, and after the thoracic cage was opened, the lungs were carefully dissected. Lungs were washed with HBSS and evaluated for the presence of tumor nodules on a scale of 1+ to 3+: Fewer than 10 nodules/lung, 1+.; 10–20 nodules/lung, 2+; more than 20 nodules/lung, 3+ or 'significant tumor burden'. Then, one lung was fixed in formalin, paraffin-embedded, and stained with hematoxylin and eosin. RencaHA nodules were explanted from the remaining lung and a single-cell suspension was made by mechanical dissociation and passage through nylon mesh. Explants of RencaHA obtained at different time points during tumor progression demonstrated continued expression of HA, as determined by staining with the antibody against HA, H-18 (data not shown).

**Re-isolation of clonotypic T cells after *in vivo* transfer.** On the day of analysis, spleen cells were obtained by passing them through nylon mesh and centrifugation on a Ficoll gradient (Ficoll-Paque; Pharmacia). Then, splenocytes were passed through nylon wool to enrich samples for T cells. Optimization of this technique has allowed us to obtain at least $5 \times 10^6$ highly purified T cells per spleen, an amount sufficient for our studies.

**Flow cytometric analysis.** T cells were stained with FITC-conjugated goat anti-mouse CD4 (Caltag, Burlingame, California) and biotinylated rat anti-clonotypic TCR antibody MAb 6.5, followed by PE-conjugated streptavidin (Caltag, Burlingame, California). For this analysis, 50,000 gated events were collected on a FACSCAN (Becton Dickinson, San Jose, California) and analyzed using CellQuest software (Becton Dickinson, San Jose, California). Data represent the mean + s.e.m. of the percentage of cells expressing the clonotypic TCR. Background staining of splenocytes or lymph node cells from naive BALB/c mice is usually less than 0.10%.

**Antigen-specific proliferation.** Purified T cells ($4 \times 10^4$ cells/well) from the experimental groups were mixed with fresh splenocytes ($8 \times 10^4$ cells/well) from naive BALB/c mice to which 12.5 μg/ml of synthetic HA peptide (amino acids 110–120; SFERFEIFPKE) was or was not added. The cells were pulsed with $^3$H-thymidine (1 mCi/well, Amersham) after 3 d in culture. Cells were collected 18 h later with a Packard Micromate cell harvester. Thymidine incorporation into DNA was measured as counts per minute (cpm) on a Packard Matrix 96 direct beta counter. Data represent as cpm per 100 clonotype-positive T cells

**Cytokine release.** T cells purified and plated as described above were cultured with media alone or HA peptide (12.5 μg/ml) plus fresh BALB/c splenocytes. Then, 48 h later, supernatants were collected and stored at −70 °C, then assayed for IL-2, IL-4 and IFN-γ by ELISA (R&D Systems, Minneapolis, Minnesota). Values for T cells cultured in media alone were less than 10% of the values for HA-stimulated T cells. Data represent pg/ml of the specific cytokine per 100 clonotype-positive T cells per well.

**Analysis of clonotypic T cells from draining lymph nodes.** To assess the fate and function of those clonotype-positive T cells in the regional lymph nodes, the peritracheal, peribronchial and mediastinal lymph nodes were collected from tumor-free mice and from RencaHA-bearing mice. Lymph nodes from three mice per group were pooled, and cell suspensions were made by passing the samples through nylon mesh and centrifugation on a Ficoll gradient. Between $2 \times 10^6$ and $3 \times 10^6$ lymph node cells were obtained from the pooled samples of tumor-bearing mice, and between $0.5 \times 10^6$ and $1 \times 10^6$ cells were obtained from tumor-free mice. The phenotypic and functional characteristics of these cells were analyzed as described above (flow cytometric analysis, antigen-specific proliferation and cytokine production).

**Intravenous injection of a tolerogenic dose of HA peptide$_{110\text{-}120}$.** The experimental design in Fig. 6*b* was used to evaluate the effect of antibody against CD40 treatment in a well-characterized model of peptide-induced tolerance (Fig. 5). Anti-HA/I-E$^d$ TCR$^+$ transgenic T cells ($2.5 \times 10^6$) were transferred into BALB/c mice on day 0. Half the mice received 100 μg/day of the agonist antibodies against CD40 intravenously 1 d before and 1 d after the transfer of T cells. On day +2, an intravenous injection of 275 μg purified HA peptide$_{110\text{-}120}$ was given to some mice. On day +15 after T-cell transfer, half the mice in each subgroup were immunized subcutaneously with $1 \times 10^7$ plaque-forming units of vac-HA. All the mice were killed for analysis 6 d after immunization (day +21), and T cells from the spleen were obtained. Phenotypic and functional characteristics of these reisolated T cells were evaluated as described above.

***In vivo* priming with vac-HA.** A recombinant vaccinia virus encoding hemagglutinin from the 1934 PR8 strain of influenza was a gift from F. Guarnieri. Vac-HA was amplified on Hu-TK$^-$ cells in the presence of 25 μg/ml 5-bromo-2'-deoxyuridine (Sigma). Virus was purified from the cellular lysate by sucrose banding, and titered by plaque assay on B-SC-1 cells. On the days indicated for each particular experimental design, mice were primed by subcutaneous inoculation with $1 \times 10^7$ plaque-forming units of recombinant vaccinia encoding HA suspended in 0.1 ml HBSS.

**Statistical analyses.** Two-way analysis of variance (ANOVA) was used to evaluate the magnitudes of tumor and antibody against CD40-induced effects for clonotypic T-cell expansion, proliferation and cytokine production. To compare the experimental groups in Fig. 3, we used a one-way ANOVA.

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*Review*

# Tumor Escape from Immune Surveillance

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**Abstract.** The bases for an efficient anti-tumor immune response begin to be better defined. Nonetheless, neoplastic cells develop various strategies to escape immune surveillance, which are discussed here in order to better design the therapeutic possibilities of immune manipulation. The absence of specific tumor antigen as well as the weak expression of major histocompatibility complex (MHC) molecules hinder the recognition of the neoplastic cells by T lymphocytes. The defect of expression by the tumor of the ligands for the T cell activation costimulatory molecules is particularly harmful for the immune response since it induces tolerance. Finally, tumor cells can inactivate effector T lymphocytes through the secretion of inhibitory cytokines, induction of apoptosis or functional inactivation. The multiplicity of the means to oppose an effective anti-tumor response challenges the adaptative mechanisms of the immune system. For example, the natural killer cells target tumor cells not expressing MHC class I molecules. Numerous possibilities of tumor immunogenicity restoration have been demonstrated at least *in vitro*, such as stimulation of the cancerous cells by CD40 or cytokine treatment, which could lead to several promising therapeutical approaches.

**Key words:** cancer; immunodeficiency; immunotherapy; lymphocyte.

The immune system is a complex and highly regulated defense mechanism which preserves the integrity of the organism by the elimination of all elements considered as "non-self" or "modified self". The development of cancer constitutes a potentially lethal aggression to the host. Does the immune system participate efficiently to tumor elimination? Clinical data answer at least partly to this question, via analyzes of immunocompromised patient populations and of the immunotherapy approaches already used against cancer.

A highly increased frequency of cancers is observed during the course of congenital immunodeficiency syndromes such as the X-linked immunodeficiency syndrome[1, 15, 30, 41] or the common variable hypogammaglobulinemia[14]. Another example of immunodeficiency corresponds to organ transplantation which requires a strong immunosuppression because of discrepancy between donor and receiver MHC. Therapeutic immunosuppression relies on the utilisation of drugs such as cyclosporin and of corticosteroids, which are powerful inhibitors of T lymphocyte functions. Lymphoma incidence ranges from 1% in renal transplantation to 8% in pulmonary transplantation, and correlates with the intensity of the immunosuppression[52]. In this setting,

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Abbreviations used: MHC – major histocompatibility complex, HIV – human immunodeficiency virus, CML – chronic myeloid leukemia, AML – acute myeloid leukemia, GVH – graft versus host, GVL – graft versus leukemia, APCs – antigen presenting cells, TcR – T cell receptor, IL-2 – interleukin 2, CLL – chronic lymphocytic leukemia, IFN – interferon, NK – natural killer, TNFR – tumor necrosis factor receptor, FL – follicular lymphoma.

the best first line treatment is the reduction of the immunosuppression which usually results in the total disappearance or regression of the lesions. More recently, the HIV epidemic is responsible for an increase in the frequency of many cancers, more particularly systemic or cerebral lymphoma, Kaposi's sarcoma and cervical cancer of the uterus. These data show that immunodeficiency, either congenital, induced by viral infection or related to therapy, favour the development of cancer, especially of lymphoma.

In addition to these data, allogeneic bone marrow or peripheral stem cell transplantation support the existence of an anti-tumor resonse at least in the case of the malignant hemopathies, such as chronic (CML) and acute (AML) myeloid leukemia. Indeed, donor T lymphocytes recognise the recipient organism as "non-self", a phenomenon called "graft-versus-host" (GVH) reaction. In order to decrease this potentially lethal reaction, attempts of T lymphocyte graft depletion[16] or functional inactivation by anti-IL-2 receptor antibodies have been performed[5]. This graft depletion resulted in reduced incidence and severity of GVH, but also in a very important increase in leukemic relapses. This suggests the existence of graft-versus-leukemia (GVL) reaction mediated by the T lymphocytes. This hypothesis is further supported by the efficiency of the donor T lymphocyte infusion in the treatment of AML or CML relapses[28, 56, 57].

Once demonstrated the role of immune system in the antitumor response, and before considering the different mechanisms of tumor escape, we will remind the mechanisms leading to the specific recognition of an antigen by T lymphocytes. First, the antigen (Ag) is degraded in peptides which reach the antigen presenting cell (APC) surface presented by the MHC class I (endogenous peptides) or class II (exogenous peptides) molecules. When a T lymphocyte meets an APC, multiple links between the two cells are created through adhesion/costimulation molecules, which transmit an activation signal to the T cell. Then, the Ag/MHC molecule complex is presented to the specific receptor CD3/TcR. If the Ag presented to the T lymphocyte corresponds to its antigenic specificity, an activation signal is transmitted via the CD3/TcR. The lymphocyte receives therefore two signals, one by the adhesion/costimulation molecules and the other by the Ag receptor, leading to its proliferation, the secretion of the numerous cytokines necessary for the amplification of the immune response – interleukin-2 (IL-2) in particular – and/or to cytotoxic properties. At each step of the immune response, dysfunctions can favor tumor escape.

## Absence of Specific Antigen

Various tumor Ags with different levels of specificity have been described. Many neoplasms have chromosomal anomalies leading to the generation of fusion genes potentially transcribed as proteins. One example is the bcr/abl fusion in CML[7], which is expressed only in tumor cells. In addition to this paradigm of specific tumor Ag, differentiation Ags, such as tyrosinase in melanoma, are transiently expressed in normal cells during their ontogeny[3]. Tumor induced by virus often express some viral Ags, like for example peptides of the oncoprotein E7 of the HPV16 virus in cervical uterus cancer[42]. From these examples it appears that the absence of tumor Ag is not, in many cases, the right explanation for an absence of or inefficient anti-tumor immune response.

## Defect of Expression of MHC Molecules and of Antigenic Peptide Transport

As previously shown, antigenic peptide presentation by MHC molecules at the surface of APCs (class I for $CD8^+$ cytotoxic lymphocytes, class II for the $CD4^+$ auxiliary T cells) is necessary for their recognition by effector T lymphocytes. The class I MHC molecules consist of a membrane $\alpha$-chain associated to a soluble $\beta$-chain, the $\beta$-2-microglobuline. The loss of the $\beta$-chain is responsible for the absence of expression of the $\alpha$-chain at the APC surface, impairing the antigenic recognition. Many examples of loss or reduction of expression of the class I MHC molecules have been described in various tumors, such as head and neck carcinoma[11], prostate cancer[46], small cell lung cancer[29, 43], Burkitt's lymphoma[45], renal or colic carcinomas[24, 25]. This expression of the class I molecules can be highly heterogeneous within the primitive tumor. For example, MHC class I molecule expression in melanoma clones is variable and correlates to their level of recognition by cytolytic lymphocytes[44]. Still in melanoma, loss of expression of MHC class I molecules can occur after immunotherapy[44]. The most immediate interpretation of this observation is that immunotherapy leads to a better recognition, and therefore destruction, of cells expressing MHC molecules, but is not able to eliminate the class I negative clones. Loss of expression of MHC class I molecules, in regard to the primary tumor, is also frequently observed in metastases[11, 26].

### Defect of Expression of Adhesion/Costimulatory Molecules

Several couples of adhesion/costimulation molecules and their ligands such as LFA-1-ICAM-1, CD2-LFA-3 or CTLA-4/CD28-B7-1/B7-2 play an important role in the immune response. We will focus on this last system, which is central in tumor immune recognition[20, 37, 38, 48]. The engagement of CD28 at the lymphocyte surface with its ligands B7-1 or B7-2 at the tumor cell surface provides to the T lymphocyte the second signal necessary to reach complete activation and IL-2 synthesis, in order to avoid anergy development. Solid tumors does not express B7-1 or B7-2[20]. The situation is more complex in hematological neoplasms. The follicular and diffuse large cell lymphoma express B7-1 and B7-2 but at a very weak level, insufficient to allow efficient allogeneic immune recognition[13]. The so-called mantle-cell lymphoma or small lymphocytic lymphoma, as well as chronic lymphocytic leukemia (CLL), do not express B7-1 or B7-2[13, 39, 55]. On the other hand, B7-1 is expressed at the surface of Reed-Sternberg cells in Hodgkin's disease, and contributes to their immune recognition[12, 33]. In AMLs, B7-1 is in general very little expressed whereas B7-2 is readily present in particular in myelo/monocytic subtypes[10, 23, 54]. The regulation of these molecules can also be aberrant. For example, the stimulation by interferon γ (IFN-γ) does not induce the expression of B7 molecules in the myelo/monocytic AMLs, in contrast to their "normal counterpart", the monocyte[10]. If the complete absence of B7 molecules does not allow the development of the immune response, some deleterious effects may also result from very low expression level. Indeed, the CTLA-4 molecule binds, like CD28, to B7-1 and B7-2, but has two major differences with regard to CD28: a higher affinity[31] and the delivery of an inhibitory signal for the lymphocyte functions when CD28 is not committed at the same time[17]. If very few B7 molecules are available at the tumor cell surface, CTLA-4 may be stimulated without CD28 engagement, delivering therefore a message of negative regulation to the immune effector cells.

### Tumor Cell Counterattack

Malignant cells can destroy or inactivate the effector cells, either by the secretion of soluble molecules (cytokines or soluble receptors) or by direct cellular contacts. The best illustration of this phenomenon is the "Fas counterattack"[50]. Briefly, the Fas molecule, when stimulated by his ligand (FasL), induces a message of active cell death or apoptosis. The Fas molecule is expressed by many cells, in particular by T lymphocytes. An expression of a functional FasL by tumor cells is frequent in colic carcinoma[35], hepatoma[51], melanoma[21] or lymphoma[58]. Consequently, tumor infiltrating lymphocytes can be destroyed by apoptosis, whereas the tumor itself is often at least partially resistant to Fas-dependent apoptosis[40]. The existence of increased circulating levels of soluble FasL in some hematological malignancies such as large granular lymphocyte lymphoma or natural killer (NK) lymphoma has been demonstrated[53]. Other mechanisms of lymphocyte function inactivation have also been described, such as the inhibition of the CD40/CD40L system. The CD40 molecule is a member of the superfamily of tumor necrosis factor receptors (TNFR) and is expressed on many types of tumor cells[4, 19]. The CD40/CD40L system plays a central role in the development of the immune response, establishing a reciprocal dialogue between T lymphocytes and the different types of APCs. The engagement of CD3/TcR by antigenic peptides presented by MHC quickly induces CD40L expression at the T lymphocyte surface. Then, CD40L binds to CD40 and induces or increases the expression at the tumor cell surface of various adhesion/costimulation molecules, such as B7-1, B7-2, LFA-3 or ICAM-1, which provide the second signal required to activate the naive T cells, amplify the immune response and prevent the induction of tolerance. Blood and splenic $CD4^+$ lymphocytes from patients with CLL fail to express CD40L after activation by CD3[6]. The co-incubation of B cells from CLL patients with allogeneic T lymphocytes induces reduction of CD40L expression at the T cell surface. These data suggest therefore that CLL B lymphocytes inhibit the immune response by the suppression of the CD40-triggered T lymphocyte stimulation. Among the indirect mechanisms, the secretion by the tumor cells prostaglandin E2, transforming growth factor β, interleukin-10 or many other cytokines may contribute to T cell function inhibition[34].

### How Can the Immune Response Bypass the Tumor Escape Mechanisms?

The immune system can also react in order to inhibit tumor development. Among the most interesting mechanisms are the so-called NK activity. The NK cells participate in the innate response against viruses, bacteria or tumor cells but, in contrast with B cells or T cells, without expressing Ag specific receptors (im-

munoglobulins or TcR) and without MHC restriction. The mechanism explaining the action of these cells is supported on the contrary by the "missing self hypothesis"[32]. Schematically, NK cells present on their surface receptors for MHC class I molecule, whose binding induces inactivation of their cytolytic functions. In the absence – or alteration – of MHC class I at the tumor cell membrane, the NK cells are not inactivated and therefore destroy the target cell. Finally, the neoplastic cells are kept between two antitumor mechanisms; if they express class I MHC molecules, they are susceptible to be destroyed by MHC-restricted specific T lymphocytes, while, if they lose their MHC class I determinants they become potential targets for the NK cells.

Various therapeutic interventions could contribute to improve the antitumor response, in particular by the increase of expression of adhesion molecules on the tumor surface. For example, the utilization of IL-2 in the AMLs increases blast cell expression of ICAM-1 and LFA-3[36]. The IFNs[22], but also some other cytokines still not used in human therapy such as IL-4[9] or IL-7[8] can also improve the immune recognition of the transformed cells. Recently, cancer cell stimulation by CD40 was shown to be highly efficient to restore immune response against weakly immunogenic tumors such as the follicular lymphoma (FL). The stimulation of FL cells by CD40 increases the expression of the adhesion/costimulation molecules B7-1, B7-2, ICAM-1 or LFA-3, and re-establish their recognition by allogeneic T lymphocytes[47]. Once alloreactive T lymphocytes have been primed by the tumor cells stimulated by CD40, they are also capable of efficient recognition and destruction of cells from the same lymphoma even not stimulated by CD40[47]. In addition, the reactivity and the possibilities of expansion of the tumor-infiltrating lymphocytes is greatly increased if they are incubated with FL pre-stimulated by CD40[49]. Another potentially favourable effect of tumor cell triggering via CD40 is the induction of the expression MHC molecules as well as restoration of a functional antigenic peptide transport which both favour Ag presentation and recognition[27]. The stimulation by CD40 could therefore be employed like an alternative strategy in order to increase the Ag-specific MHC-restricted antitumor response[27] in particular when other immunoregulatory cytokines such as IFNs are ineffective for instance in the Burkitt's lymphoma[2]. Finally, an original mechanism by which the stimulation via CD40 could improve the immune recognition is the induction of cytokine secretion by tumor cells. For example, CD40 stimulation of Reed-Sternberg cells in Hodgkin's disease induces them to secrete IL-8, IL-6, or TNF, which may play a role in the modulation of the immune response[18] via chemoattraction and activation of monocytes or T lymphocytes, in addition to direct antitumor effects.

## Perspectives

This overview has summarized some of the mechanisms used by tumor in order to escape the immune response, and the putative therapeutic possibilities to improve it. Nonetheless, some important mechanisms have not been discussed: a tumor growth potential exceeding the cytotoxic capacities of the T lymphocytes, tumor heterogeneity or its inaccessibility to the immune effectors, the variability in antigenic evolution, the resistance of the cancerous cells to the cytolysis, the limitation of T cell repertoire by the lymphocyte depletion induced by chemotherapy or radioterapy. All these data would ideally be considered in immunotherapy protocols in order to optimise their efficiency.

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# Dendritic Cell Secretion of IL-15 Is Induced by Recombinant huCD40LT and Augments the Stimulation of Antigen-Specific Cytolytic T Cells[1]

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**Dendritic cells (DC) are professional antigen-presenting cells which stimulate strong proliferative and cytolytic T cell responses. Stimulation of CD40 on dendritic cells by its ligands and anti-CD40 antibodies induces maturation and enhances DC stimulatory ability. In order to understand the mechanism by which ligand:CD40 interactions augment DC function, we assessed the role of T cell stimulatory cytokines IL-12 and IL-15 in the function of DC stimulated with soluble trimeric CD40L, a recombinant fusion protein incorporating three covalently linked extracellular CD40L domains (huCD40LT). Peripheral blood derived DC treated with huCD40LT and/or IFN-γ were used to stimulate T cell responses *in vitro* to specific antigens. DC treated with huCD40LT or IFN-γ/huCD40LT stimulated enhanced T cell proliferation to CASTA, a soluble protein from *C. albicans*, induced T cells with augmented antigen-specific lysis, and increased the yield of antigen-specific IFN-γ-producing T cells. IL-15 production by DC was enhanced in cultures treated with huCD40LT and correlated with expansion of antigen-specific cytolytic T cells. Addition of a neutralizing anti-IL-15 monoclonal antibody inhibited the expansion of viral and tumor antigen-specific T cells stimulated by IFN-γ and huCD40LT-treated DC. In contrast, this enhanced stimulatory ability of DC did not appear to depend on synthesis of IL-12 since huCD40LT treatment stimulated the generation of antigen-specific cytokine producing and cytolytic T cells without increased IL-12 production. Addition of anti-IL-12 monoclonal antibody did not inhibit expansion of these cells. These data suggest that production of IL-15 but not IL-12 is an important factor in the enhanced immunostimulatory ability of huCD40LT-treated DC.** © 1999 Academic Press

*Key Words:* **dendritic cells; IL-15; CD40L; tumor antigens; antigen presentation.**

## INTRODUCTION

The dendritic cell is a professional antigen presenting cell (APC)[2] which induces the formation of antigen-specific immune responses in naive T cells (1, 2). DC-mediated immune responses include the development of primary and secondary T cell helper and cytolytic immune responses, T-cell-dependent antibody production, and induction of tolerance (1–4). Vaccination with peptide-pulsed DC has been shown to induce anti-viral and anti-tumor T cell responses in mice and causes regression of established tumors (5, 6). DC capture and process antigen, become activated in tertiary lymphoid tissue, migrate to secondary lymphoid tissues, and stimulate T-cell-dependent immune responses (1, 7, 8). DC exposed to LPS and proinflammatory cytokines mature, demonstrate decreased capacity for new antigen presentation, increase their expression of immunomodulatory cell surface markers, and have enhanced ability to stimulate immune responses (9–13). Tumor cell-induced defects in DC maturation and function have been reported (14) and may be due to tumor cell production of vascular endothelial growth factor (15). DC have been shown to produce a variety of cytokines during maturation which may be important for their immune activating function, including IL-12 and IL-15 (16–19).

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[2] Abbreviations used: DC, dendritic cell; APC, antigen presenting cell; PBMC, peripheral blood mononuclear cells; IFN-γ, interferon-gamma; LPS, lipopolysaccharide; NK, natural killer; CTL, cytolytic T cell; MLR, mixed lymphocyte reaction; TNF, tumor necrosis factor; IL-, interleukin; huCD40LT, soluble trimeric CD40 ligand fusion protein; DMSO, dimethyl sulfoxide; PBS, phosphate-buffered saline; TBS, Tris-buffered saline; GM-CSF, granulocyte-macrophage colony stimulation factor; CASTA, Candida skin test antigen; HLA, human leukocyte antigen; IL–15, interleukin.

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IL-12, a heterodimeric 70-kDa cytokine, is an important mediator in the establishment of both antigen-specific T cell and nonspecific NK cell immune responses (16, 20, 21). Dendritic cell (17, 18) production of IL-12 in response to bacterial products and inflammatory mediators may be important for the stimulation of IFN-γ-producing cells in antigen-specific cytolytic T cell responses (18, 22–24) and has been hypothesized to play a role in mature DC stimulation of allogeneic proliferative and cytolytic immune responses (25).

IL-15 is a recently identified cytokine which is functionally similar to IL-2 and stimulates both T and NK cells (26–28). IL-15 has been demonstrated to increase HIV-specific CTL *in vitro* (29) and is associated with increased T cell response to the intracellular pathogen *M. leprae* (30). IL-15 can act as a chemoattractant for T cells and induces effector mechanisms in both cytolytic T cells and NK cells (26, 28, 31). IL-15 is produced by dendritic cells after exposure to bacterial products and induction of phagocytosis (19, 32) but not by T cells.

The interaction between cell surface protein CD40 on antigen presenting cells and its ligand gp39 is critical to the development of T-cell-dependent humoral immune responses (33, 34). The importance of this interaction in the generation of cell-mediated and humoral immunity is well documented (35, 36), and recent data suggest that CD40/CD40L interactions play an important role in DC-induced T cell activation (24, 37). In this work we show that treatment of DC with recombinant huCD40LT and other immunostimulatory molecules promotes the expansion of antigen-specific CTL from normal donors and patients with melanoma. The mechanism by which peptide-pulsed DC treated with huCD40LT stimulated antigen-specific reactivity has been explored by analysis of DC cytokine production and its correlation with the immunostimulatory ability of mature DC.

## RESULTS

*Increased proliferation by melanoma patient PBMC in response to CASTA pulsed dendritic cells treated with huCD40LT.* Dendritic cells derived from $CD34^+$ progenitors (38) or peripheral blood mononuclear cell precursors (9, 39) have been grown from normal human donors in the presence of human or fetal calf serum with TNF-α and GM-CSF or IL-4 and GM-CSF, respectively. In our studies, DC from normal donors and melanoma patients were expanded in AIM-V serum-free medium supplemented with IL-4 and GM-CSF (DC) resulting in cultures which were 75 to 95% HLA-DR and CD86 positive and CD14 and CD19 negative. DC from normal donors and melanoma patients had characteristic dendritic morphology and demonstrated no significant differences in expression patterns of the aforementioned surface proteins (data not shown).

Membrane-bound CD40L and other immunostimulatory molecules have been shown to augment alloreactive proliferation stimulated by DC (24, 37), therefore we expected that DC treatment with recombinant huCD40LT would enhance the proliferation of autologous PBMC in response to soluble foreign antigens. We assayed the ability of DC treated with huCD40LT +/− IFN-γ to stimulate proliferation in response to CASTA, a *Candida albicans* protein extract known to stimulate good DTH responses (40) (Fig. 1). DC treated with huCD40LT stimulated a significant increase in proliferation at 72 h in response to CASTA in all normal donors and patients when compared to DC grown in GM-CSF and IL-4 (control DC). In all six melanoma patients as well as two of the three normal donors (ND2 and ND3), addition of IFN-γ enhanced proliferation beyond that stimulated by huCD40LT-treated DC. Exposure to DC to IFN-γ alone did not significantly alter proliferation in normal donors or melanoma patients. These findings suggest that huCD40LT alone or combined with IFN-γ augments the capacity of DC form normal donor and melanoma patient PBMC to stimulate proliferative T cell responses to a soluble protein.

*Development of Flu antigen-specific CTL is augmented by dendritic cells treated with huCD40LT.* Since exposure of DC to CD40L-expressing cells also enhanced the ability of DC to stimulate allogeneic cytotoxicity (25) we hypothesized that synthetic recombinant immunomodulatory proteins like huCD40LT would increase the capability to stimulate MHC Class I restricted CTL responses. To test this hypothesis 9-day DC were pulsed with the HLA-A2 restricted Flu-M1 peptide (NP 66-75), then treated with IFN-γ and/or huCD40LT, and used to stimulate autologous PBMC from $HLA\text{-}A2^+$ normal donors (Figs. 2 and 3). Ten days after primary *in vitro* stimulation with DC treated with huCD40LT +/−-IFN-γ autologous PBMC effector cells demonstrated antigen-specific cytotoxicity (42 and 47%, respectively, at E:T ratio of 30:1) while effector cells stimulated with DC exposed to IFN-γ or grown in GM-CSF and IL-4 (control DC) induced less than 25% specific lysis (Fig. 2A). Background lysis of unpulsed targets was less than 10% at 30:1 E:T ratios. After a second *in vitro* stimulation, peptide-pulsed DC from all treatment groups stimulated comparable levels of antigen-specific lysis (33–39%) at 30:1 E:T ratio (Fig. 2B). Identical effector cells stimulated with control DC or DC treated with huCD40LT, IFN-γ, or the combination of huCD40LT and IFN-γ were used in an IFN-γ ELISPOT assay to determine whether the enhanced cytotoxicity correlated with the number of Flu-M1-specific IFN-γ-producing T cells (Fig. 3). Treatment of DC with huCD40LT (1850 +/− 125) or IFN-γ plus huCD40LT (2310 +/− 629) resulted in significant increases of Flu-M1-specific IFN-γ-producing effectors

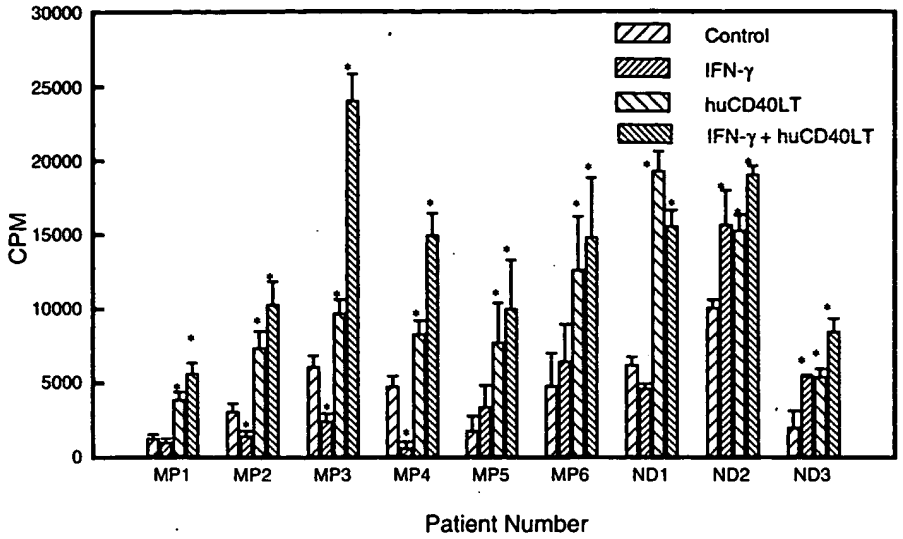


**FIG. 1.** huCD40LT enhances DC-induced proliferation to CASTA. DC from six melanoma patients (MP1–MP6) and three normal donors (ND1–ND3) were treated with IFN-γ, huCD40LT, or IFN-γ/huCD40LT and pulsed overnight with 10 μg/ml CASTA. DC were harvested and used as stimulators of autologous PBMC in a 72-h proliferation assay. Proliferation was measured by tritiated thymidine incorporation. Conditions resulting in changes in proliferation which are significantly different from control for each patient are denoted with *. Statistical significance was established using Dunnett's method of multiple comparisons at the level of $P < 0.05$ (46) and a one-way analysis of variance of the logarithm transformation of all cpm measurements. Proliferation is reported as mean CPM of five replicate wells above background with 95% confidence intervals.

compared with control DC (33 +/− 106). Exposure to IFN-γ alone (480 +/− 50) yielded a modest increase in stimulation compared with control DC. Addition of anti-CD40 antibody M2 to huCD40LT and IFN-γ-treated DC blocked the enhanced effector cell production (data not shown). These results indicate that huCD40LT treatment resulted in DC from normal donors with enhanced ability to stimulate antigen-specific cytolytic and IFN-γ-secreting T cells after a single *in vitro* stimulation compared with control or IFN-γ-treated DC.

*huCD40LT treatment of dendritic cells stimulates tumor antigen-specific CTL from melanoma patients.* To test whether treatment of patient DC with IFN-γ and huCD40LT resulted in stimulation of increased

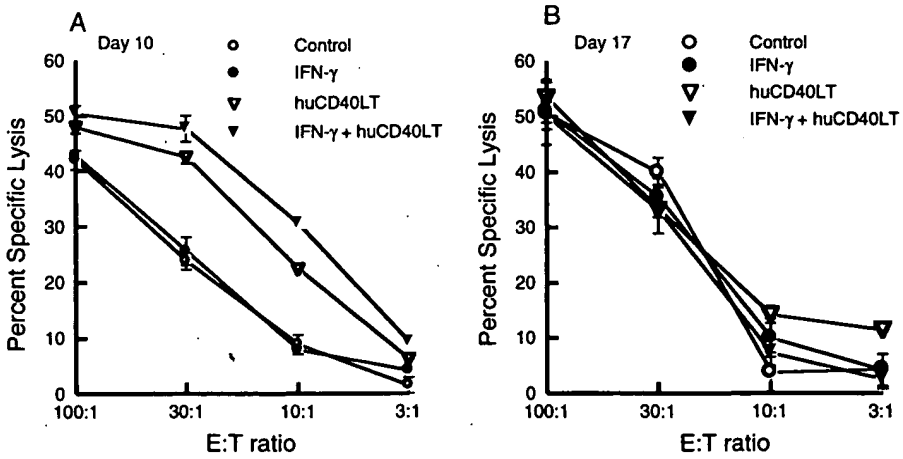


**FIG. 2.** huCD40LT stimulates DC-induced viral antigen-specific lysis. DC were treated with IFN-γ or huCD40LT and pulsed overnight with Flu-M1 peptide as described under Materials and Methods. DC were harvested and used as stimulators of autologous PBMC for antigen-specific CTL growth. Flu-M1 antigen-specific effector cells were grown for 10 days following primary *in vitro* stimulation (A) or 17 days after receiving a second *in vitro* stimulation with untreated DC on day 10 (B). Cultures were harvested and the isolated effector cells were assayed for their ability to induce Flu-M1 specific lysis. Specific lysis was determined by percentage lysis of $^{51}$Cr-labeled Flu-M1 peptide-pulsed T2 cells minus percentage lysis of gp100 peptide-pulsed T2 cells, which did not exceed 10% at 30:1 E:T ratio. Representative data are presented from experiments which have been repeated in two normal donors.

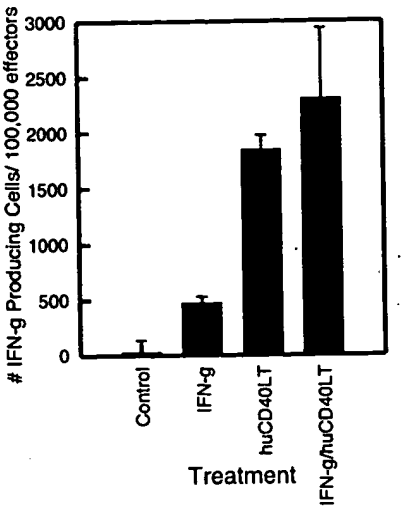


FIG. 3. huCD40LT enhances DC-induced expansion of viral antigen-specific IFN-γ-producing cells. DC were treated with IFN-γ or huCD40LT and pulsed overnight with Flu-M1 peptide as described under Materials and Methods. DC were harvested and used as stimulators of autologous PBMC for antigen-specific CTL growth. Flu-M1 antigen-specific effector cells were grown for 10 days following primary *in vitro* stimulation. Cultures were harvested and the isolated effector cells were assayed for number of IFN-γ-producing cells per 100,000 effectors as described under Materials and Methods. Values represent the number of IFN-γ-positive cells responding to Flu-M1 peptide-pulsed T2 cells minus percentage lysis of gp100 peptide-pulsed T2 cells. Representative data are presented from experiments which have been repeated in four normal donors and one melanoma patient.

tumor antigen-specific CTL activity, MART-1 peptide-pulsed DC treated with IFN-γ, huCD40LT, or the combination were used to stimulate autologous melanoma patient PBMC. As with Flu-M1, MART-1 peptide-pulsed DC treated with huCD40LT alone or IFN-γ and huCD40LT stimulated antigen-specific cytotoxicity after 9 days (17 and 23%, respectively, at 100:1 E:T ratio) (Fig. 4A). Background lysis of unpulsed targets was less than 5% at all E:T ratios tested. Effectors stimulated with IFN-γ-treated or untreated DC did not show significant MART-1 specific cytotoxicity above background 9 days after stimulation. Identical effector cells stimulated with control, huCD40LT-, IFN-γ, or huCD40LT/IFN-γ-treated patient DC were used in an IFN-γ ELISPOT assay to confirm that enhanced cytotoxicity correlated with the number of MART-1-specific IFN-γ-producing T cells (Fig. 4B). Treatment of DC cultures with huCD40LT (960 +/− 45) or IFN-γ and huCD40LT (906 +/− 45) but not IFN-γ alone (320 +/− 92) resulted in greater than a fourfold increase of MART-1-specific IFN-γ-producing effectors compared with control DC (200 +/− 60). To understand the mechanism of the increased immunostimulatory ability of peptide-antigen-pulsed DC treated with huCD40LT we examined the production of cytokines implicated in the formation of T-cell-mediated immune responses by DC treated with huCD40LT with or without IFN-γ.

*Exposure of DC to huCD40LT alone stimulates increased IL-15 production.* In order to determine whether IL-15, a T cell stimulatory molecule secreted by DC (19, 32), contributed to the enhanced stimulation of antigen-specific CTL by huCD40LT-treated DC, supernatants were harvested at 24-h timepoints after day 10 medium change of control, huCD40LT-, and/or IFN-γ-treated DC, concentrated fivefold, and assayed for IL-15 production (Fig. 5A). Peak IL-15 production by DC was in the 0- to 24-h supernatant and increased threefold after treatment with huCD40LT (58 pg/ml) or IFN-γ/huCD40LT (97 pg/ml) but not in response to treatment with IFN-γ (15 pg/ml) alone compared with control DC (10 pg/ml). Addition of anti-CD40 antibody abrogated the increase in IL-15 production by huCD40LT- and IFN-γ-treated DC (data not shown). IL-15 production by DC treated with huCD40LT with or without IFN-γ correlated with the increased antigen-specific immunostimulatory ability of DC and was seen with DC from normal donors and patients.

*IFN-γ plus huCD40LT but not huCD40LT alone induces increased synthesis of IL-12 from peripheral blood-derived DC.* DC grown from PBMC-derived monocytes of normal individuals have been shown to produce IL-12 in response to treatment with J558L cells expressing membrane-bound CD40L. In those studies DC production of IL-12, a cytokine which has been shown to enhance cell-mediated immune responses (16, 20, 21), correlated with an increased ability to generate alloreactive proliferative and cytolytic responses (24). To determine whether IL-12 is produced by DC grown with and without huCD40LT and/or IFN-γ, DC supernatants from melanoma patients and normal donors were harvested from 4 to 120 h after replacement of AIM V medium on day 10 of culture. Control, IFN-γ, huCD40LT, or IFN-γ/huCD40LT DC were assayed for IL-12 production by ELISA (Fig. 5B). These data indicate that DC cultures derived from different melanoma patient or healthy donor PBMC produce IL-12 only in response to the combination of a trimeric huCD40LT fusion protein and IFN-γ (187 pg/ml) but not to either agent alone (<6 pg/ml) and that IL-12 production rapidly declines without further stimulation during the initial 12 h after washout.

*Neutralizing antibodies to IL-12 and IL-15 block DC-induced proliferation to CASTA.* To determine the impact of IL-12 and IL-15 production by DC on the enhanced proliferation demonstrated by IFN-γ/huCD40LT-treated CASTA-pulsed DC we measured the effect of IL-12 and IL-15 neutralization on 72-h proliferation to CASTA (Fig. 6). Addition of a neutralizing anti-IL-12 monoclonal antibody resulted in

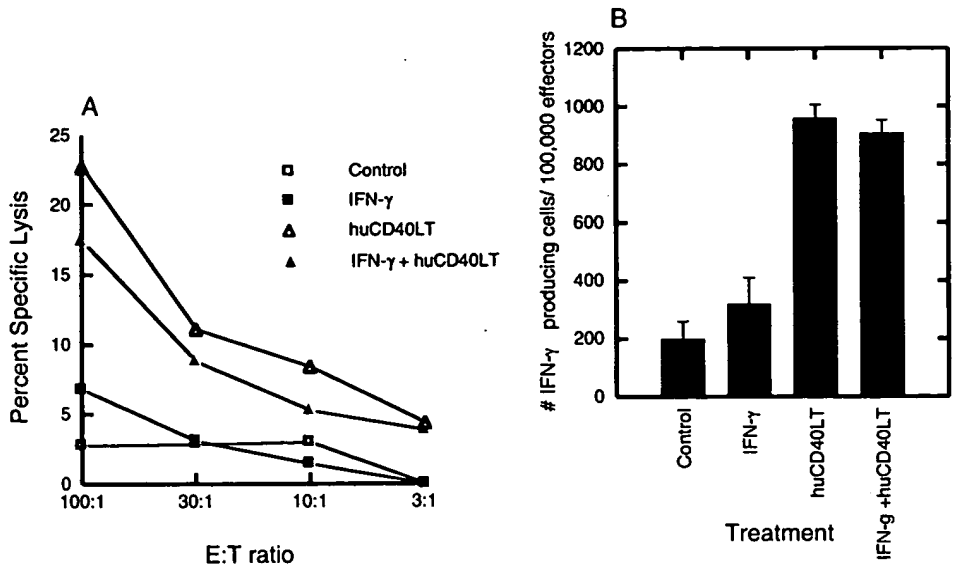


**FIG. 4.** Soluble CD40L fusion protein alone or in combination with IFN-γ-treated DC increases MART-1 tumor antigen-specific CTL. Melanoma patient DC were treated with IFN-γ, huCD40LT, or IFN-γ and huCD40LT and pulsed overnight with MART-1 peptide. DC were harvested and used as stimulators of autologous PBMC for antigen-specific CTL growth. MART-1 antigen-specific effector cells were grown for 9 days following primary *in vitro* stimulation with DC. (A) Tumor antigen-specific lysis was determined by chromium release from $^{51}$Cr-labeled MART-1 peptide-pulsed T2 target cells minus percentage lysis of gp100 peptide-pulsed T2 cells, which did not exceed 5% at any E:T ratio as described under Materials and Methods. (B) Number of effector cells which specifically responded to the MART-1 tumor antigen with production of IFN-γ was assayed by ELISPOT assay as described under Materials and Methods. Values represent the number of IFN-γ-positive cells responding to MART-1 peptide-pulsed T2 cells minus percentage lysis of gp100 peptide-pulsed T2 cells. Representative data are presented from an experiment which has been repeated in four patients.

reduction of the average tritiated thymidine incorporation in two normal donors by 40 +/− 9%, anti-IL-15 antibody reduced proliferation minimally by 20 +/− 10%, and both anti-IL-12 and anti-IL-15 reduced proliferation by 55 +/− 2.5% while an isotype control antibody did not alter proliferation. These data suggest that IL-12 but not IL-15 plays a role in DC-mediated proliferation to CASTA, a soluble *C. albicans* protein.

*An anti-IL-15 but not an anti-IL-12 monoclonal antibody blocks the expansion of HLA-A2-restricted antigen-specific T cells stimulated by IFN-γ/huCD40LT-treated DC.* To examine whether production of IL-15 and/or IL-12 by DC is responsible for the enhancement of tumor antigen-specific CTL activity we blocked IFN-γ/huCD40LT-treated DC-stimulated CTL cultures using anti-IL-15 and anti-IL-12 monoclonal antibodies and measured the number of IFN-γ-producing T cells in an ELISPOT assay. Blocking antibodies as well as an isotype control antibody were added to appropriate wells at the establishment of the CTL cultures. Ten days later MART-1- (Fig. 7A) or Flu-M1- (Fig. 7B) specific T cells were measured. Treatment of MART-1 peptide-pulsed DC with IFN-γ/huCD40LT resulted in an increase in the number of specific IFN-γ-producing cells from 90 +/− 55 (control DC) to 650 +/− 86. Addition of anti-IL-15 blocking antibody decreased the number of IFN-γ-producing cells to 120 +/− 75 while addition of anti-IL-12 antibody and an isotype control antibody did not significantly block expansion of antigen-specific IFN-γ-producing cells (493 +/− 63 and 530 +/− 11, respectively). Similarly, treatment of Flu-M1 peptide-pulsed DC with IFN-γ/huCD40LT resulted in an increase in the number of specific IFN-γ-producing cells from 33 +/− 106 (control DC) to 2310 +/− 629. Addition of anti-IL-15 blocking antibody decreased the number of IFN-γ-producing cells to 825 +/− 214 while addition of anti-IL-12 antibody and an isotype control antibody did not significantly block expansion of Flu-M1 antigen-specific IFN-γ-producing cells (1947 +/− 268 and 2607 +/− 548, respectively). Addition of neutralizing anti-IL-15 antibody to control DC did not decrease effector cell stimulation (data not shown).

In order to determine if IL-15 produced by DC resulted in the expansion of antigen-specific T cells with cytolytic activity, effectors were also tested for their ability to lyse appropriate target cells in a chromium release assay, shown in Fig. 8. Treatment of the Flu-M1 peptide-pulsed DC with IFN-γ/huCD40LT resulted in an increase in the Flu-specific lysis from 23% (control DC) to 47% at a 30:1 E:T ratio. Addition of anti-IL-15 blocking antibody decreased Flu-M1 specific lysis to 12% at a 30:1 E:T

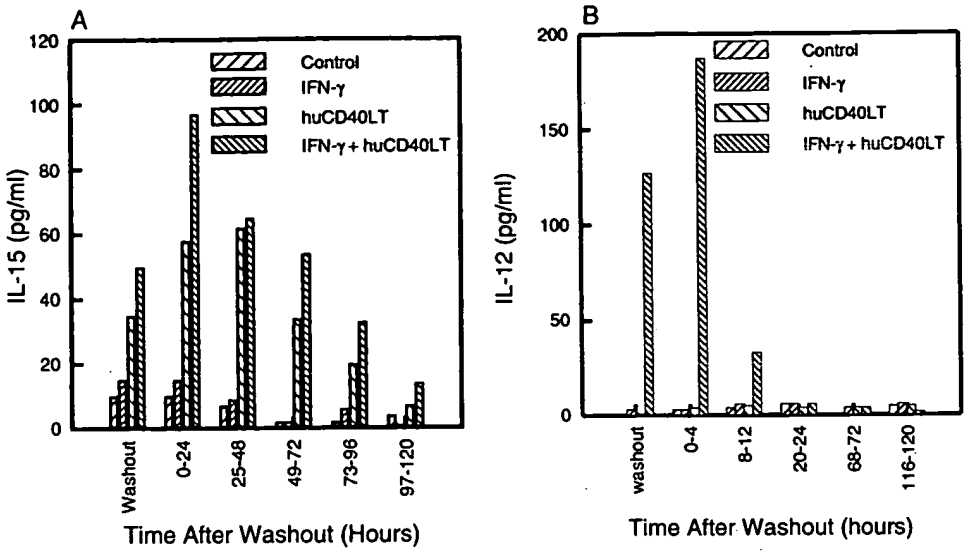


FIG. 5. IL-12 and IL-15 production by DC is enhanced by immunomodulatory agents. DC were grown for 9 days and then exposed to IFN-γ, TNF-α, huCD40LT, and IFN-γ/huCD40LT for 18 h. Supernatants were collected as described and assayed by sandwich ELISA for the presence of IL-15 (A) or IL-12 (B). IL-15 release in A is from fivefold concentrated supernatants collected every 24 h after the day-10 medium change. IL-12 release in B are supernatants collected from 4 to 120 h after day-10 medium change. Representative data are presented from an experiment which has been repeated in four patients and two normal donors.

ratio while addition of anti-IL-12 neutralizing antibody and an isotype control antibody did not significantly block Flu-M1 specific lysis (49 and 43%, respectively, at a 30:1 E:T ratio). Background lysis of unpulsed targets was less than 10% at 30:1 E:T ratios. These data suggest that increased production of IL-15 contributes to huCD40LT/IFN-γ-treated DC stimulation of tumor and viral antigen-specific T cells from normal donors and cancer patients.

## DISCUSSION

Cella *et al.* established that monocyte-derived DC cocultured with J558L cells expressing CD40L stimulated increased levels of proliferation in an allogeneic MLR (24). In this study, we have employed a construct which covalently links three CD40L extracellular domains in one recombinant molecule to stimulate human dendritic cells. The role of DC in the generation of antigen-specific immune responses has been the focus of extensive study, and published data suggest that DC have the ability to prime effective viral and tumor antigen-specific immunity (6, 41). APC capable of stimulating tumor antigen-specific immune responses *in vitro* might facilitate the generation of anti-tumor immune responses *in vivo* after adoptive transfer (6, 41). The melanoma antigen MART-1 (aa27-35) (42, 43) and the viral-specific antigen Flu-M1 (aa66-75) (44), encoding 9 amino acid HLA-A2-restricted epitopes, were chosen as model antigens for assessment of MHC Class I restricted immune responses, and CASTA, a *C. albicans* protein which generates potent DTH responses were used for assessment of proliferative responses to soluble protein. MART-1 responses were tested in melanoma patients and Flu-M1 responses in normal donors and patients. Our studies demonstrate that recombinant huCD40LT induces enhanced DC-stimulated proliferation in response to CASTA in normal donors and melanoma patients. The addition of IFN-γ to huCD40LT-treated DC resulted in increased mean proliferation to CASTA.

Our studies also demonstrate that addition of recombinant huCD40LT enhanced the ability of peptide-pulsed DC to stimulate the expansion of antigen-specific cytotoxic and IFN-γ-producing T lymphocytes from autologous PBMC *in vitro*. This immunostimulatory effect of huCD40LT permitted the detection of antigen-specific CTL within 10 days of primary *in vitro* stimulation of the PBMC as had been demonstrated for allogeneic CTL (25). In contrast to the effect of huCD40LT upon CASTA-induced proliferation, treatment of peptide-pulsed DC with huCD40LT stimulated similar levels of antigen-specific cytotoxicity with or without IFN-γ. These data show that treatment of DC with huCD40LT resulted in the production of APC with increased capacity for stimulation of viral and tumor antigen-specific immune responses.

Production of cytokines by APC is an important mechanism for the generation and orientation of immune responses. IL-15 and IL-12 are produced by activated macrophages and dendritic cells and their

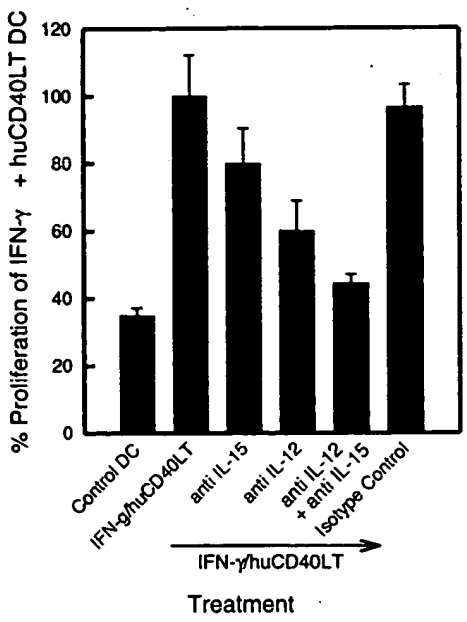


**FIG. 6.** Neutralization of IL-12 and IL-15 decreases proliferation of autologous PBMC stimulated by CASTA-pulsed DC. DC from normal donors were treated with IFN-γ and huCD40LT and pulsed overnight with CASTA and used to stimulate proliferation by autologous PBMC. Proliferation assays were treated with anti-IL-15, anti-IL-12, or a control $IgG_1$ monoclonal antibody. Proliferation was measured by tritiated thymidine incorporation and is reported as percentage of IFN-γ + huCD40LT-treated DC-stimulated control. Reported values represent the average +/− 95% confidence intervals of a representative experiment performed in two normal donors.

synthesis is enhanced in response to bacterial products and by induced phagocytosis (17, 19, 21, 24, 28). Production of IL-15 by antigen presenting cells is important for the generation of cell-mediated immune responses (29, 30) and IL-15 production by DC has been demonstrated to enhance chemotaxis of T cells (16–19). IL-15 binds to a receptor composed of the β and γ chains of the IL-2 receptor to exert its function which overlaps the actions of IL-2. IL-15 is induced by DC after phagocytosis or in response to LPS, suggesting that it plays an early role in the induction and amplification of cell responses. Its chemotactic effects when produced by DC would be consistent with a role in early T cell activation. In our experiments ligation of CD40 on DC by recombinant huCD40LT with or without IFN-γ resulted in a threefold increase in IL-15 production. In order to test the hypothesis that IL-15 plays a role in DC stimulation of MHC Class I restricted T cell responses, we demonstrated that IL-15 but not IL-12 neutralization in DC-stimulated T cell cultures abrogated the expansion of antigen-specific IFN-γ-producing and cytolytic T cells from normal donors and melanoma patients. We concluded that there is a direct link between IL-15 production by DC and the stimulation of MHC Class I restricted antigen-specific T cells and that production of IL-15 can be stimulated by ligation of CD40 on DC *in vitro* by a recombinant CD40L trimer fusion protein. IL-15 may enhance DC-mediated stimulation of effector T cells through direct activation of effector mechanisms in an IL-2-like role or alternatively enhance chemotaxis of T cells.

Monocyte-derived DC (MODC) isolated from the peripheral blood of normal donors have been shown to produce IL-12 in response to coculture with cells expressing membrane-bound CD40L on J558 transfectants (24, 25). In our study DC did not produce IL-12 in response to ligation by recombinant huCD40LT but required additional stimulation by IFN-γ. This difference from published data could be attributed to at least four different factors: (1) the CD40L-expressing cells in the literature are producing IFN-γ, (2) interactions other than CD40/CD40L between the DC and the J558 cell may modulate the activation of DC, (3) recombinant huCD40LT fusion protein may not induce the same effects as aggregated membrane-bound CD40L, and (4) the different media and culture conditions used in our studies resulted in DC in a different activation state than the MODC produced by Cella *et al.* Our data demonstrate that while IL-12 production by DC correlated with increased proliferation in response to CASTA, it did not coincide with production of antigen-specific cytolysis or IFN-γ-secreting MHC Class I restricted antigen-specific T cells. Furthermore, IL-12 neutralization decreased autologous proliferation in response to CASTA but had no effect on MART-1 or Flu-M1 antigen-specific effector T cell expansion. However, exogenous IL-12 has been shown to increase the production of cytolytic immune responses stimulated by DC (data not shown) and to the stimulation of CASTA-dependent proliferation. IL-12 produced by DC may be responsible for the stimulation of NK or $CD4^+$ T cells which may aid in the stimulation of antigen-specific CTL. Maturation of DC with huCD40LT may bypass the requirement for $CD4^+$ and NK cell help in the expansion of class I restricted CTL. This could explain the delay observed in CTL activation by untreated DC until after a second restimulation. Studies investigating the role of IL-15, IL-12, and other cytokines in the stimulation of cell-mediated responses augmented by MHC Class II help are being performed in our laboratory.

In this study, we have also demonstrated that dendritic cells from normal donors or patients with melanoma can stimulate potent anti-viral or anti-tumor T cell responses *in vitro* which are amplified by recombinant huCD40LT and depend on production of IL-15. This result suggests that viral or tumor antigen peptide-pulsed dendritic cells grown from normals or melanoma patient PBMC treated with huCD40LT and

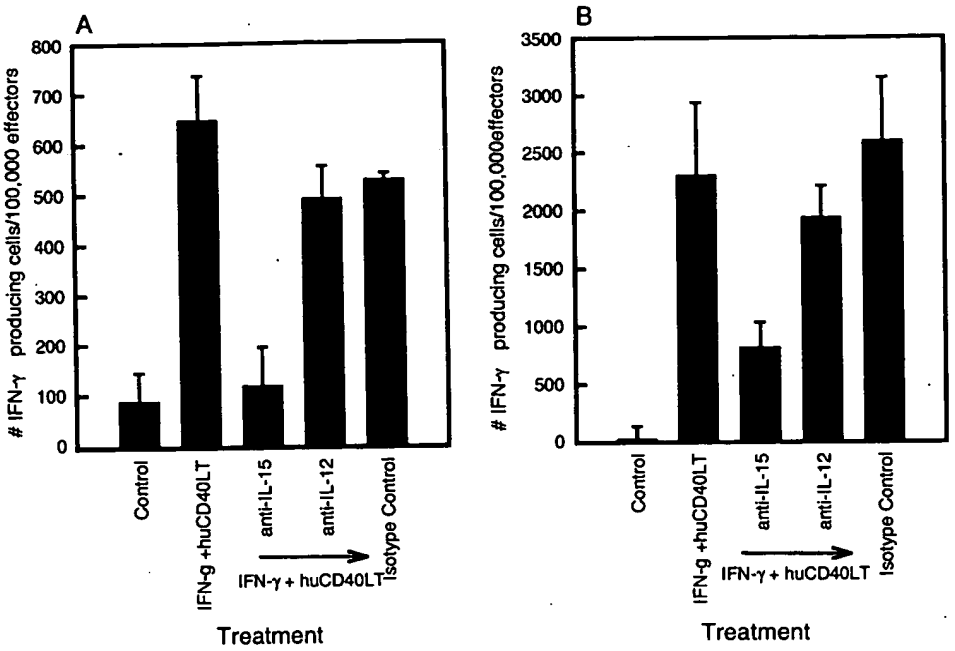


**FIG. 7.** An anti-IL-15 but not an anti-IL-12 neutralizing monoclonal antibody inhibits the expansion of antigen-specific IFN-γ-producing cells by IFN-γ/huCD40LT-treated DC. DC were treated with IFN-γ and huCD40LT and pulsed overnight with MART-1 (A) or Flu-M1 (B) peptide. DC were harvested and used as stimulators of autologous PBMC for antigen-specific CTL growth. IFN-γ/huCD40LT-treated DC-stimulated CTL cultures were treated with anti-IL-15, anti-IL-12, or a control $IgG_1$ monoclonal antibody. Antigen-specific effector cells were grown for 9 days following primary *in vitro* stimulation with DC. The number of antigen-specific IFN-γ-producing cells per 100,000 effector cells was assayed by ELISPOT assay as described under Materials and Methods. Values represent the number of IFN-γ-positive cells responding to MART-1 or Flu-M1 peptide-pulsed T2 cells minus those responding to gp100 peptide-pulsed T2 cells. Representative data are presented from an experiment which has been repeated for MART-1 in three patients and Flu-M1 in four normal donors and one melanoma patient.

expressing IL-15 may be effective as a vaccine to augment anti-viral or anti-tumor immunity. This preclinical idea will be tested in an upcoming clinical trial at our institution in patients with melanoma.

## MATERIALS AND METHODS

*Collection and purification of mononuclear cells.* Peripheral blood mononuclear cells were isolated from leucopheresis specimens of HLA-$A2^+$ normal donors or melanoma patients who had their tumor resected and were rendered free of detectable disease. PBMC were enriched by Ficoll–Hypaque density gradient purification and aliquoted and frozen at $5 \times 10^7$ cells/ml in 50% AIM V medium (Gibco, Grand Island, NY), 40% heat-inactivated human AB sera (Omega Scientific, Irvine, CA), and 10% DMSO (Sigma, St. Louis, MO). Cell yields were greater than $3 \times 10^9$ PBMC per leucopheresis.

*Cytokines, cell lines, and reagents.* Recombinant IL-4 ($6.35 \times 10^7$ IU/mg) and GM-CSF ($1.35 \times 10^8$ IU/mg) used for the production of DC were kindly provided by Dr. Sathwant Narula, Schering-Plough Research Institute (Kenilworth, NJ). Recombinant soluble CD40L trimeric fusion protein was generously provided by Immunex Corporation (Seattle, WA). Recombinant human IFN-γ was kindly provided by Medarex Corporation (Annandale, NJ). Recombinant human IL-2 was generously provided by Chiron Therapeutics (Emeryville, CA). Recombinant human IL-7 was kindly provided by Sanofi Pharmaceuticals (Labege, France). Antigenic peptides MART-$1^{27-35}$ (AAGIGILTV) (45) and Flu-$M1^{58-66}$ (GILGFVFTL) (44) were synthesized on a solid state peptide synthesis machine at the USC/Norris Cancer Center microchemical core synthesis facility and reconstituted in 100% DMSO (10 mg/ml). CASTA, a mixture of Candida-associated proteins, was purchased from Greer Laboratories (Lenoir, NC) (40). T2 cells expressing HLA A2.1 were a kind gift of Dr. Franco Marincola, NCI.

*Growth of dendritic cells.* Dendritic cells were prepared from peripheral blood mononuclear cells by a modification of the method of Romani *et al.* (39). PBMC were thawed and allowed to adhere to plastic for 1 h. Nonadherent cells were removed with agitation. Adherent cells were grown in AIM V medium (Gibco) containing GM-CSF (800 U/ml) and IL-4 (1000 U/ml) (AIM V-DC medium) for 10 days receiving fresh medium and cytokine on day 7. DC cultures were exposed to IFN-γ (250 U/ml), huCD40LT (1.5

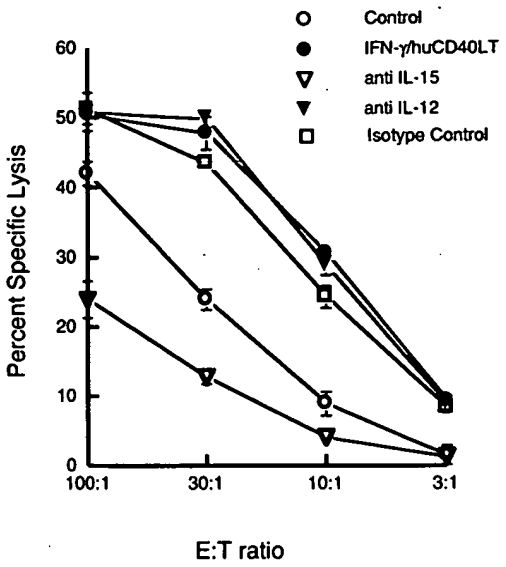


**FIG. 8.** An anti-IL-15 but not an anti-IL-12 neutralizing monoclonal antibody inhibits the expansion of antigen-specific CTL by IFN-γ/huCD40LT-treated DC. DC were treated with IFN-γ and huCD40LT, pulsed overnight with Flu-M1 peptide, and used to stimulate autologous PBMC for antigen-specific CTL growth. IFN-γ/huCD40LT-treated DC-stimulated CTL cultures were treated with anti-IL-15, anti-IL-12, or a control $IgG_1$ monoclonal antibody. Tumor antigen-specific lysis was determined by chromium release from $^{51}Cr$-labeled Flu-M1 peptide-pulsed T2 cells minus percentage lysis of gp100 peptide-pulsed T2 cells, which did not exceed 10% at 30:1 E:T ratio as described under Materials and Methods. Representative data are presented from an experiment which has been repeated in two normal donors.

μg/ml), and/or TNF-α (75 U/ml) for the last 18 h of 10-day cultures.

*Proliferation assay.* DC were pulsed with 10 μg/mL antigen 20 h prior to harvest and IFN-γ, huCD40LT, or IFN-γ and huCD40LT were added 18 h prior to harvest. DC stimulators ($2.5 \times 10^4$) were irradiated with 6000 R and incubated with $1.5 \times 10^5$ PBMC responders in 96-well plates for 72 h. Tritiated thymidine (1 μCi) (NEN, Boston, MA) was added to each well for the last 18 h of the assay. Cells were harvested onto glass fiber filters using a Model 12010 cell harvester (Skatron, Sterling, VA) and counted in a Model C1600 liquid scintillation counter (Packard Instruments, Palo Alto, CA). Statistical significance was established using Dunnett's method of multiple comparisons at the level of $P < 0.05$ (46) and a one-way analysis of variance of the logarithm transformation of all cpm measurements. Proliferation is reported as mean cpm of five replicate wells above background with 95% confidence intervals. Anti-IL-12 (15 μg/ml) and anti-IL-15 (10 μg/ml) neutralizing monoclonal antibodies or an isotype control antibody were added at the induction of each proliferation assay for blocking studies.

*Cytokine ELISA.* IL-12 (p70) and IL-15 production by DC cultures was measured by sandwich ELISA according to the manufacturer's specifications (R&D Systems, Minneapolis, MN). Dendritic cells were cultured in AIM V-DC medium for 10 days to a density of $10^6$ cells in 2 ml of medium per well of a 6-well plate. On day 9 IFN-γ and huCD40LT were added to specified cultures. On day 10 culture supernatants were removed and replaced with 2 ml of fresh AIM-V medium. Supernatant samples were harvested at the indicated time points, then aliquoted and frozen at −80°C until analysis. IL-15 supernatants were fivefold concentrated prior to analysis using microcon 10-spin filters (Amicon, Beverly, MA).

*Growth of antigen-specific CTL.* DC cultures were grown in T-75 tissue culture flasks to a density of $5 \times 10^6$ cells/flask in the presence or absence of cytokine or huCD40LT for the last 18 h of culture. On day 9 of culture at least 30 min prior to adding immunomodulatory agents, DC were pulsed with 10 μg/mL of either Flu-$M1^{58-66}$ or MART-$1^{27-35}$ peptide. On day 10 DC cultures were irradiated (6000 rad) and medium was removed and replaced with AIM-V medium containing $5 \times 10^7$ PBMC responder cells. All CTL cultures received IL-7 (10 ng/ml) at the establishment of culture and IL-7 (10 ng/ml) and IL-2 (25 IU/ml) on day 5 of culture. CTL were grown for 10 days prior to assay or they were restimulated with peptide-pulsed adherent PBMC on day 10 and harvested for assay on day 17. Blocking of IL-15 and IL-12 was performed by addition of neutralizing monoclonal antibodies, M112 (10 μg/ml) (Genzyme, Cambridge, MA) and C8.6 (15 μg/ml) (Pharmingen, San Diego, CA), respectively, at the establishment of antigen-specific CTL cultures. Isotype control was the $IgG_1$ 107.3 antibody (Pharmingen).

*Cytotoxicity assay.* After 9 or 17 days in culture, graded doses of effectors were plated in 96-well round-bottom plates with 5000 T2 target cells incubated overnight with either antigen-specific or an irrelevant control peptide and labeled with $^{51}Cr$ (Amersham, Arlington Heights, IL). After 4 h, supernatants were harvested using a harvesting frame (Skatron) and released chromium-labeled protein was measured using a gamma counter (Packard Instruments). Percentage of antigen-specific lysis was determined by subtracting the percentage of lysis with irrelevant HLA-A2 restricted peptide-pulsed T2 targets from the percentage of lysis with antigen peptide-pulsed T2 targets.

*ELISPOT assay.* IFN-γ ELISPOT assays were performed using a modification of a protocol established by Fujihashi *et al.* (47). On day 1 mouse anti-human IFN-γ capture antibody NIB42 (10 μg/ml) (Pharmingen) was aliquoted into MAHA S4510

plates (Millipore Corporation, NY) and incubated for 18 h at room temperature. On day 2 the supernatant was removed, and blocking buffer (RPMI with 10% fetal bovine sera) (Omega Scientific) was added and allowed to incubate at 37°C for 1 h. Blocking buffer was removed and replaced with graded doses of effector cells and $10^5$ T2 cells pulsed overnight at 37°C with either antigen-specific or irrelevant peptide. On day 3 the plates were washed three times with PBS and three times with PBS containing 0.05% Tween 20 followed by overnight incubation at 4°C with a biotin-labeled anti-human IFN-$\gamma$ detection antibody 4S.B3 (2.5 $\mu$g/ml) (Pharmingen). On day 4 the biotin-conjugated antibody was removed and plates were washed with TBS followed by a 1-h incubation at 37°C with streptavidin–alkaline phosphatase (1:2000 in TBS) (Gibco, Gaithersburg, MD). Plates were washed three times with TBS. BCIP/NBT color solution (Kirkegaard-Parry Laboratories, Gaithersburg, MD) was added and allowed to incubate for 15–25 min. IFN-$\gamma$-producing cells/well were enumerated using a SZH stereo zoom microscope as well as an automated ELISPOT reader (Scientific Products, Los Angeles, CA).

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IMMUNOBIOLOGY

# CD40-Activated B-Cell Chronic Lymphocytic Leukemia Cells for Tumor Immunotherapy: Stimulation of Allogeneic Versus Autologous T Cells Generates Different Types of Effector Cells

By Raymund Buhmann, Annette Nolte, Doreen Westhaus, Bertold Emmerich, and Michael Hallek

**Although spontaneous remissions may rarely occur in B-cell chronic lymphocytic leukemia (B-CLL), T cells do generally not develop a clinically significant response against B-CLL cells. Because this T-cell anergy against B-CLL cells may be caused by the inability of B-CLL cells to present tumor-antigens efficiently, we examined the possibility of upregulating critical costimulatory (B7-1 and B7-2) and adhesion molecules (ICAM-1 and LFA-3) on B-CLL cells to improve antigen presentation. The stimulation of B-CLL cells via CD40 by culture on CD40L expressing feeder cells induced a strong upregulation of costimulatory and adhesion molecules and turned the B-CLL cells into efficient antigen-presenting cells (APCs). CD40-activated B-CLL (CD40-CLL) cells stimulated the proliferation of both CD4$^+$ and CD8$^+$ T cells. Interestingly, stimulation of allogeneic versus autologous T cells resulted in the expansion of different effector populations. Allogeneic CD40-CLL cells allowed for the expansion of specific CD8$^+$ cytolytic T cells (CTL). In marked contrast, autologous CD40-CLL cells did not induce a relevant CTL response, but rather stimulated a CD4$^+$, Th1-like T-cell population that expressed high levels of CD40L and released interferon-γ in response to stimulation by CD40-CLL cells. Together, these results support the view that CD40 activation of B-CLL cells might reverse T-cell anergy against the neoplastic cell clone, although the character of the immune response depends on the major histocompatibility complex (MHC) background on which the CLL or tumor antigens are presented. These findings may have important implications for the design of cellular immunotherapies for B-CLL.**


CHRONIC LYMPHOCYTIC leukemia of B-cell origin (B-CLL) is the most common type of leukemia in the western hemisphere. Despite a continued effort to improve the outcome of B-CLL by new chemotherapeutic agents, the disease remains uncurable. Therefore, it seems rewarding to evaluate alternative treatment options such as immunotherapy.

Although some cases of spontaneous remission in CLL have been reported,[1] B-CLL cells generally fail to induce a clinically relevant immune response. Whereas the clinical appearance of B-CLL often remains stable for years, the total tumor cell burden tends to expand at variable speed without any apparent reaction of the immune system against the tumor. This may be caused by an impaired T-cell–mediated immune response,[2] including a depressed function of natural killer (NK) cells and antibody-mediated cellular cytoxicity (ADCC)[3,4]; a reduced susceptibility of the leukemic cells towards the effector cells[5]; or an inability of the neoplastic cells to function efficiently as antigen-presenting cells (APCs), similar to other lymphoid malignancies.[6,7]

In the specific case of B-CLL, the malignant cells are the neoplastic counterpart of a subpopulation of CD5$^+$ B cells[8,9] that might function as professional APCs and effectively present endogenous tumor antigens to T cells. Moreover, the B-CLL specific idiotype provides a unique tumor antigen that might be recognized by the immune system, similar to other lymphoid malignancies in which this strategy has been tested successfully in clinical trials.[10-12] However, despite their strong expression of major histocompatibility complex class I (MHC I) and class II (MHC II) molecules, B-CLL cells are generally ineffective stimulator cells in mixed lymphocyte reactions.[13]

It has been demonstrated that expression of MHC molecules is not sufficient for the induction of a productive immune response and that adequate expression of adhesion and costimulatory molecules is critical to stimulate a potent T-cell response. Failure to receive these signals renders potential effector T cells anerg or tolerant.[14] Among the costimulatory molecules, the B7 family appears to be unique, because it has been demonstrated that B7-1 (CD80) and B7-2 (CD86) are both necessary and sufficient to prevent the induction of anergy.[15-17] In normal and malignant B cells, activation of CD40 seems to be a major stimulus for the induction of B7-1 and B7-2.[7,18]

In this study we sought to determine whether B-CLL cells could be turned into efficient APCs and whether this could reverse T-cell anergy against the neoplastic cell clone. Stimulation of CD40 on B-CLL cells in vitro induced a strong upregulation of adhesion and costimulatory molecules. Repeated stimulation of allogeneic versus autologous T cells with CD40-CLL cells resulted in different effector populations. Allogeneic CD40-CLL cells activated CD8$^+$, cytolytic T cells with activity against both native B-CLL and CD40-CLL cells. In marked contrast, autologous CD40-CLL activated predominantly CD4$^+$ T cells that had no major cytolytic activity. The results suggest that cellular vaccination studies in B-CLL may use at least two different strategies that depend on the source of T cells: when using allogeneic, peripheral blood T cells from healthy donors, CD40-CLL cells are very potent in expanding CD8$^+$, cytolytic T cells in vitro; this potential might be used for adoptive immune transfer studies. In contrast, autologous T

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cells respond to CD40-CLL cells primarily by an expansion of $CD4^+$ Th1-like T cells; this might be exploited and further studied in a trial in which CD40-CLL cells are directly applied to the patient.

## MATERIALS AND METHODS

*Patients.* After informed consent, peripheral blood samples were obtained from patients with B-CLL. The diagnosis of B-CLL was based on standard clinical and laboratory criteria.[19] The study included 12 patients (4 women and 8 men; 49 to 82 years of age). Staging was performed according to the Binet classification.[20] Characteristics of the patients studied are summarized in Table 1.

*HeLa/SF cells transfected with CD40L cDNA.* The CD40L coding region was amplified as previously described.[21] Briefly, RNA was isolated from activated human T cells. Reverse transcription was followed by a two-step polymerase chain reaction (PCR). The first-step PCR was performed using sense primers coding for the first 20 nucleotides of the CD40L coding sequence and antisense primers coding for the last 23 nucleotides of the CD40L coding sequence. In a second step, the amplified PCR products reamplified using extended primers. The sense primer (5′-GTA GGA ATT CGT CGA CGC CGC CAC CAT GAT CGA AAC ATA CAA CC-3′) contains *Eco*RI and *Sal* I sites, a strong translational start site, and the first 20 nucleotides of the CD40L coding sequence. The antisense primer (5′-GAC TAG TGT CGA CGA ATT CAG AGT TTG AGT AAG CCA AAG-3′) contains the last 23 nucleotides of the CD40L coding sequence, including the stop codon and *Eco*RI, *Sal* I, and *Spe* I sites. For each step, 20 cycles were performed (95°C for 1 minute, 48°C for 30 seconds, and 72°C for 1 minute), followed by one cycle at 72°C for 10 minutes. The 0.8-kb PCR product was digested with *Eco*RI, gel-purified, and ligated into *Eco*RI-digested pcDNA3.1 vector (purchased from Invitrogen, NV Leek, The Netherlands). Plasmids containing the CD40L insert were transfected via electroporation in HeLa/SF cells. Transfectants were selected by growth in 250 μg/mL G418 and further subcloned. Biologic activity was determined by costimulation of B-cell proliferation and differentiation.

*Isolation of B-CLL cells.* Mononuclear cells (MNCs) from peripheral blood samples were isolated by centrifugation on a Ficoll/Hypaque (Seromed, Berlin, Germany) density gradient and depleted from monocytes by overnight adherence to plastic tissue culture flasks. Subsequently, the nonadherent lymphocytes were cryopreserved in liquid nitrogen in the presence of 10% dimethyl sulfoxide (DMSO; Sigma, München, Germany). As assessed by flow cytometry, greater than 95% of these cells typically coexpressed CD19 and CD5 surface molecules.

*B-CLL cell culture.* For CD40L-induced activation, freshly isolated B-CLL cells were cultured as previously described.[14] Briefly, CD40L or mock-transfected NIH3T3 fibroblasts or HeLa/SF cells were γ-irradiated at 200 Gy, plated at $5 \times 10^5$ cells/well in 6-well plates in media without G418, and incubated overnight at 37°C in a 5% $CO_2$ humidified atmosphere. Before addition of the B-CLL cells, the feeder layers were washed twice with phosphate-buffered saline, and tumor cells were cultured at $2 \times 10^6$ cells/mL in Iscove's medium (Seromed) supplemented with 10% heat-inactivated fetal calf serum (FCS), 2 mmol/L L-glutamine, 100 U/mL penicillin, 100 μg/mL streptomycin. For further studies, culture was performed in presence or absence of interleukin-2 (IL-2; 20 IU/mL), IL-4 (1 IU/mL), and interferon γ (IFNγ; 20 IU/mL). For performing functional studies, tumor cells were harvested after 24, 72, and 120 hours of culture; purified by ficoll density gradient centrifugation; washed; and analyzed by flow cytometry. For T-cell restimulation, the activated B-CLL cells (CD40-CLL) were aliquoted and stored in liquid nitrogen. The CD40L-transfected NIH3T3 cell line was a generous gift from Dr J. Schultze (Dana-Farber Cancer Insitute, Boston, MA). With respect to their stimulatory capacity, no differences between CD40L-transfected NIH3T3 fibroblasts and CD40L-transfected HeLa/SF cells were detected; therefore, both feeder cell lines are referred to as t-CD40L throughout the manuscript.

*Cytokines and cytokine measurements.* Recombinant human IL-2 (rhIL-2), rhIL-4, and rhIFNγ were obtained from Boehringer Mannheim (Mannheim, Germany) and used as indicated. Cytokine measurements were performed using commercial IL-4 and IFNγ enzyme-linked immunosorbent assay (ELISA) kits (R&D Systems, Wiesbaden-Nordenstadt, Germany) according to the manufacturer's instructions. The detection limits of the assays were 5 to 2,000 pg/mL.

*Immunophenotyping.* Immunophenotyping was performed with the following monoclonal antibodies (MoAbs) conjugated with fluorescein isothiocyanate (FITC), phycoerythrin (PE), or phycoerythrin cyanine 5 (PE-Cy5): CD3, CD4, CD5, CD8, CD19, CD20, CD23, CD25, CD28, CD54, CD56, CD58, CD69, CD95, HLA-ABC, HLA-DR, anti-κ, anti-λ (Coulter/Immunotech, Hamburg, Germany), CD40, CD40L, CD80, CD86, and CD95L (PharMingen, Hamburg, Germany). Fluorescence was measured with a Coulter Epics XL-MCL (Coulter Electronics, Miami, FL).

*Purification of T cells.* Purification of T cells was performed by negative selection. Briefly, the monocyte-deprived MNCs from healthy donors or B-CLL patients were stained with a cocktail of antibodies (CD11b, CD16, CD19, CD36, and CD56), labeled with goat anti-rat IgG microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany), and selected using magnetic separation columns (Miltenyi Biotec). Isolated cells were greater than 95% pure as determined by immunofluorescent flow cytometry analysis (FACS) and appeared viable by exclusion of trypan blue and forward/side scatter analysis.

*Generation of effector T cells.* Purified T cells from healthy donors or B-CLL patients were stimulated with γ-irradiated (75 Gy) CD40-CLL cells or with native B-CLL cells at different effector to target (E:T) ratios ranging from 10:1 to 5:1. Stimulation was performed on days 0, 7, 14, and 21. Briefly, T cells were harvested weekly, washed, and recultured at a concentration of $1 \times 10^6$ cell/mL with γ-irradiated, autologous, or allogeneic stimulator cells in Iscove's medium (Seromed) supplemented with 5% human heat-inactivated AB-serum (Serva, Heidelberg, Germany), 2 mmol/L L-glutamine, 100 U/mL penicillin, and 100 μg/mL streptomycin. The addition of IL-2 (20 IU/mL) was perfomed 48 hours after (re)stimulation.

*Mixed lymphocyte reaction (MLR).* Irradiated (75 Gy) B-CLL cells and CD40-CLL cells were used as stimulators, cocultured at $1 \times 10^4$ cells/well in a final volume of 200 μL with allogeneic T cells at $1 \times 10^5$ cells/well in 96-well round-bottom plates, and incubated for 3 days at 37°C in a 5% $CO_2$ humidified atmosphere. The culture medium used was Iscove (Seromed) supplemented with 5% heat-inactivated human AB-serum (Serva), 2 mmol/L L-glutamine, 100 U/mL penicillin, and 100 μg/mL streptomycin. All microcultures were performed in triplicate. During the last 12 hours of the 72-hour culture period, cells were pulsed with 0.5 μCi [$^3$H] thymidine (Amersham, Braunschweig, Germany). Cells were harvested onto glass fiber filters and dried, and the [$^3$H] thymidine incorporation was measured by scintillation spectropho-

**Table 1. Clinical Features of Patients With B-CLL**

| Patient | Stage Binet | Sex/Age | Treatment |
|---|---|---|---|
| CLL-1 | A | M/60 | No |
| CLL-2 | A | M/60 | No |
| CLL-3 | C | M/68 | 6× NOSTE*, 6× IMVP16 |
| CLL-4 | A | F/72 | No |
| CLL-5 | A | F/82 | No |
| CLL-6 | C | M/59 | No |
| CLL-7 | A | M/68 | No |
| CLL-8 | A | M/77 | No |
| CLL-9 | A | M/66 | No |
| CLL-10 | C | F/61 | IFNα, 15× KNOSPE, 6× CLL-CHOP, 3× Fludarabin |
| CLL-11 | C | F/49 | 6× Fludarabin, 1× VAD, 1× IEV |
| CLL-12 | A | M/62 | No |

*Mitoxantrone/predmustine

tometry in a Wallac Microbeta Plus 1450 scintillation counter (Turku, Finland). The stimulation indexes (SI) were calculated for each individual experiment as follows: $SI = cpm_{(T\ cells + B\text{-}CLL\ cells)}/cpm_{(T\ cells)}$.

*Cytotoxicity assays.* T-cell–mediated cytotoxicity was determined using a standard 4-hour [$^{51}$Cr] release assay.[22] Unstimulated and CD40-stimulated B-CLL cells as well as NK-sensitive K562 cells were used as targets. Target cells were labeled with 100 μCi of [$^{51}$Cr] ($Na^{51}CrO_2$; Dupont, Bad Homburg, Germany) per $10^6$ cells for 2 hours at 37°C in a water bath. Thereafter, the cells were washed three times in complete medium and seeded in v-bottomed microtiter plates at a concentration of $2.5 \times 10^3$ cells/well. Indicated numbers of effector cells were added in triplicate in 200 μL of complete medium. Supernatants were collected, and the released [$^{51}$Cr] was measured in a γ-counter (LKB-Wallac 1282, Uppsala, Sweden). Spontaneous release was determined by incubation of target cells in medium alone, and maximum release was determined by resuspending the wells with 10% Triton X-100. Specific lysis was determined for each individual experiment as follows: specific lysis (%) = [(experimental [$^{51}$Cr] release − spontaneous [$^{51}$Cr] release)/(maximum [$^{51}$Cr] release − spontaneous [$^{51}$Cr] release)] × 100.

*Statistical analysis.* Differences between experimental groups were analyzed using the $\chi^2$ test and the Student's *t*-test.

## RESULTS

*B-CLL cells lack costimulatory molecules.* B7 costimulatory molecules play a crucial role in the induction of a T-cell–mediated immune response. Lack to receive costimulatory signals after antigen presentation renders T cells anergic or tolerant.[23,24] This may be a mechanism by which CLL cells escape the immune response. Therefore, we determined the cell surface expression of MHC, adhesion, and costimulatory molecules by immunophenotyping of freshly isolated B-CLL cells obtained from 12 patients. The results are summarized in Table 2. The expression level of the surface molecules on B-CLL cells was classified according to their mean fluorescence intensity (MFI). All patients tested expressed high (MFI 1.5 to 2.5 logarithm) or even very high (MFI >2.5 logarithm) levels of MHC class I and II molecules. The adhesion molecules ICAM-1 (CD54) were undetectable in 3 of 12 patients, were expressed at intermediate (MFI 0.5 to 1.5 logarithm) in 8 of 12 patients, and were expressed at high levels in 1 of 12 patients.

**Table 2. Immunophenotype of Freshly Isolated B-CLL Cells**

| | Recognition | | Adhesion | | Costimulation | | |
|---|---|---|---|---|---|---|---|
| Patient | MHC I | MHC II | ICAM-1 | LFA-3 | B7-1 | B7-2 | CD40 |
| CLL-1 | ND | +++ | − | − | − | + | + |
| CLL-2 | ND | +++ | − | − | − | + | + |
| CLL-3 | ND | +++ | − | − | − | + | + |
| CLL-4 | ND | +++ | ++ | + | − | + | ++ |
| CLL-5 | ++++ | +++ | ++ | ++ | − | ++ | ++ |
| CLL-6 | +++ | +++ | ++ | ++ | − | − | ++ |
| CLL-7 | ND | +++ | ++ | ++ | − | − | + |
| CLL-8 | +++ | +++ | ++ | ++ | + | ++ | ++ |
| CLL-9 | ++++ | +++ | ++ | ++ | + | ++ | ++ |
| CLL-10 | ++++ | +++ | +++ | ++ | + | ++ | ++ |
| CLL-11 | +++ | +++ | ++ | ++ | + | ++ | + |
| CLL-12 | ++++ | +++ | ++ | ++ | − | + | + |

Freshly isolated B-CLL cells (>95%) typically coexpressed CD19 and CD5 surface markers. Mean intensity of fluorescence: −, negative; +, >0.2-0.5 logarithm; ++, 0.5-1.5 logarithm; +++, 1.5-2.5 logarithm; ++++, >2.5 logarithm; ND, not done.

LFA-3 (CD58) was undetectable in 3 of 12 patients and was expressed at low to intermediate levels in 9 of 12 patients. The costimulatory molecule B7-1 (CD80) was not detectable in 8 of 12 patients or showed only low expression (MFI >0.2 to 0.5 logarithm) in 4 of 12 patients. B7-2 (CD86) was not expressed in 2 of 12 patients, whereas 10 of 12 patients expressed it at low to intermediate levels. CD40 was detectable in all B-CLLs (12/12) at low or intermediate levels. Together, these experiments indicated that B-CLL cells showed a markedly reduced expression of costimulatory molecules, especially of B7-1 (CD80).

*Stimulation of B-CLL cells by t-CD40L in the presence of IL-4 efficiently upregulates costimulatory molecules.* In the next step, we stimulated freshly isolated B-CLL cells by t-CD40L and mock-transfected feeder cells in the presence of IL-4 (1 IU/mL; see Materials and Methods). Stimulation by t-CD40L induced a cluster formation of B-CLL cells caused by adhesion to the stimulator cells and a hairy-cell like morphology in some of the B-CLL cells (Fig 1A and B). No aggregation or morphologic changes were observed by stimulation with mock controls. May-Grünwald-Giemsa staining of cytospin smears showed that the size of CD40-CLL cells increased due to an expansion of both nuclei and cytoplasm. Moreover, CD40-CLL showed a relatively high degree of vacuolization, corresponding to an activated state of B-lymphoid cells (Fig 1C). No plasmacytoid differentiation was seen.

To optimize the cytokine cocktail used for stimulation of B-CLL cells, B-CLL cells were stimulated by t-CD40L or mock-transfected feeder cells for 3 days in the presence of various cytokines such as IL-2 (20 IU/mL), IL-4 (1 IU/mL), and IFNγ (20 IU/mL). Figure 2A and B shows that maximal expression of B7-1 and B7-2 was induced by t-CD40L when combined with IL-4. IL-4 alone or in combination with mock stimulation only mediated a slight increase of B7-2 expression on the B-CLL cell surface (data not shown). Stimulation by t-CD40L in combination with IL-2 and IFNγ even reduced the expression of B7-1 when compared with stimulation without cytokines. The addition of IFNγ induced only an upregulation of Fas (CD95) but had no other effects (data not shown). Taken together, the combination of t-CD40L and IL-4 seemed optimal for enhancing the expression of important costimulatory molecules on B-CLL cells and was therefore used in all subsequent experiments.

To determine the optimal length of t-CD40L stimulation for full activation of B-CLL cells, time course experiments were performed. B-CLL cells were stimulated with t-CD40L for 24, 72, and 120 hours. As soon as 24 hours after stimulation by t-CD40L, a significant upregulation of ICAM-1 (CD54), LFA-3 (CD58), and Fas (CD95) was detectable (Fig 3B). Expression of costimulatory molecules B7-1 and B7-2 reached its maximum on day 3 of t-CD40L stimulation. Mock stimulation (Fig 3C) in the presence of IL-4 (1 IU/mL) caused a slight increase of the adhesion molecules ICAM-1 and LFA-3, as well as of the costimulatory molecule B7-2. No upregulation was detected for the costimulatory molecules B7-1 and Fas (CD95). Table 3 summarizes the data obtained with leukemic cells from 12 patients after a 3-day t-CD40L stimulation in the presence of IL-4 (1 IU/mL). In all cases, CD40-CLL cells showed an increased expression of B7-1 and B7-2 (Table 3). CD40

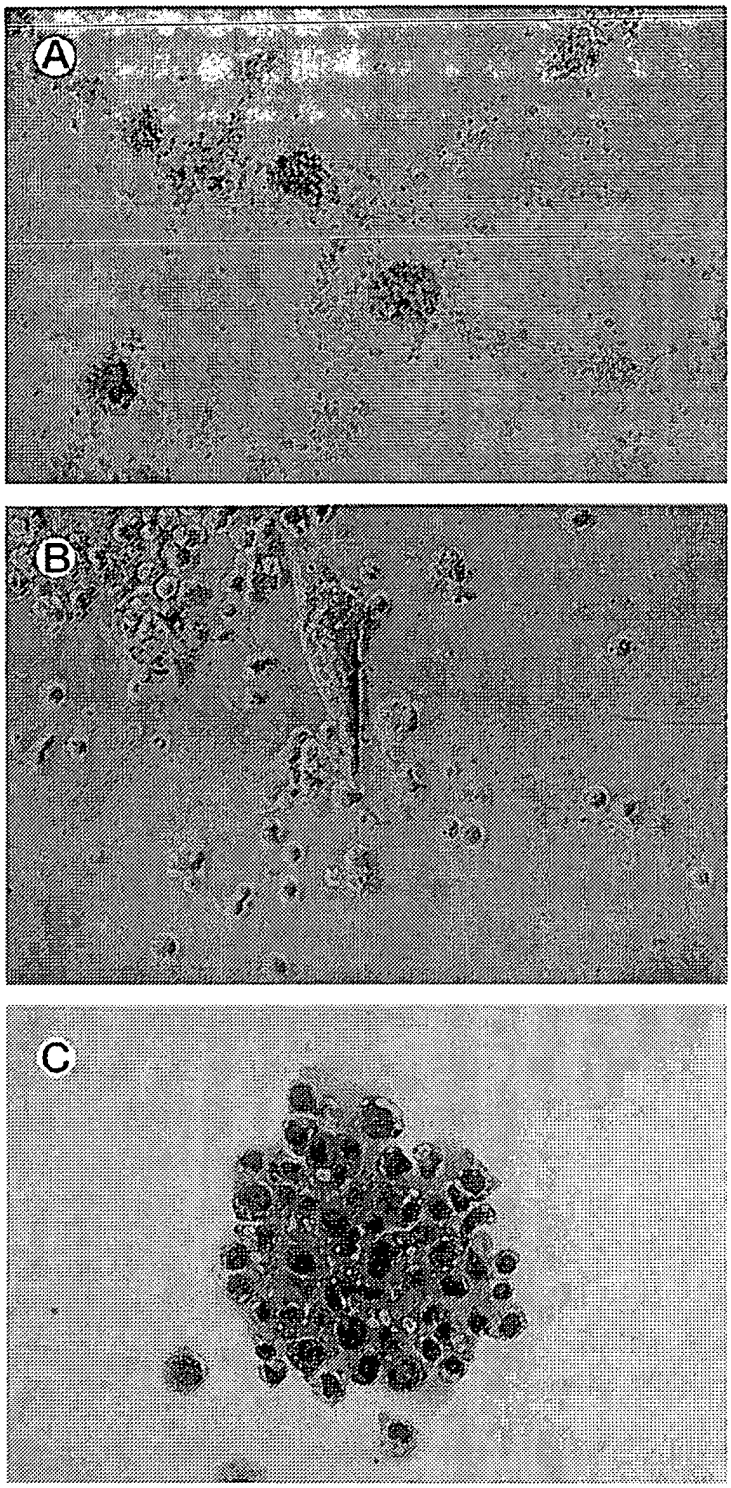


Fig 1. (A through C) Morphology of t-CD40L–stimulated CLL (CD40-CLL) cells after 3 days of culture. (A and B) Typical cluster formation of CD40-CLL cells that surround the CD40L-transfected NIH 3T3 fibroblasts (original magnifications: [A] × 100; [B] × 650). (C) CD40-CLL cells stained by May-Grünwald-Giemsa (original magnification × 650).

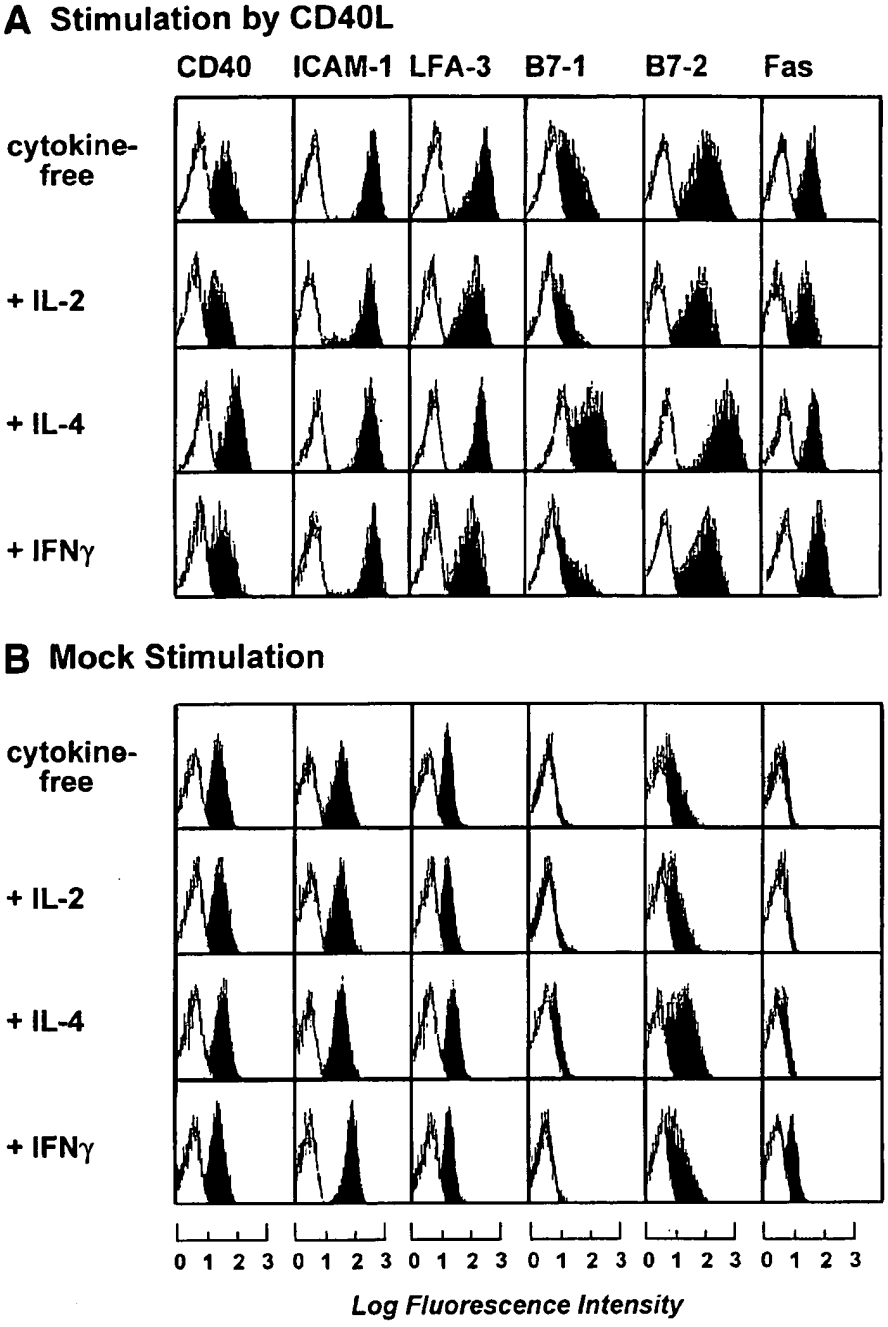


**Fig 2. Phenotypic characterization of B-CLL cells after 3 days of stimulation by CD40L or mock-transfected NIH3T3 fibroblasts in the presence of IL-2 (20 IU/mL), IL-4 (1 IU/mL), and IFNγ (20 IU/mL). Blank areas represent the isotype-matched control antibodies and the solid areas represent the fluorescence distribution of the MoAbs tested as assessed by flow cytometric analysis. The results shown are from one experiment and are representative of three independent experiments.**

expression was further increased in 8 cases. ICAM-1 and LFA-3 were also upregulated in most cases, except if they were already expressed at intermediate levels before t-CD40L stimulation (ICAM-1: patients no. 6, 7, and 10; LFA-3: patients no. 7 through 10). Expression of both MHC class I and II molecules was further increased to high or very high levels in those cases in which the expression was lower before CD40 stimulation.

*CD40L-activated B-CLL cells retain the immunophenotypic characteristics of neoplastic cells.* To exclude that treatment with t-CD40L induced the expansion of normal rather than neoplastic B cells, immunophenotypic analyses of light chain restriction and CD5 expression were performed on CD40-CLL cells. As shown in one representative experiment, CD40-CLL cells showed the phenotype of B-CLL cells as demonstrated by coexpression of CD5 and CD19 and by light chain restriction (Fig 4).

*CD40-CLL cells induce a proliferative T-cell response.* We then investigated whether CD40-CLL cells provided a proliferative stimulus to T cells. For this purpose, we used highly (>95%) purified allogeneic T cells and incubated them in the

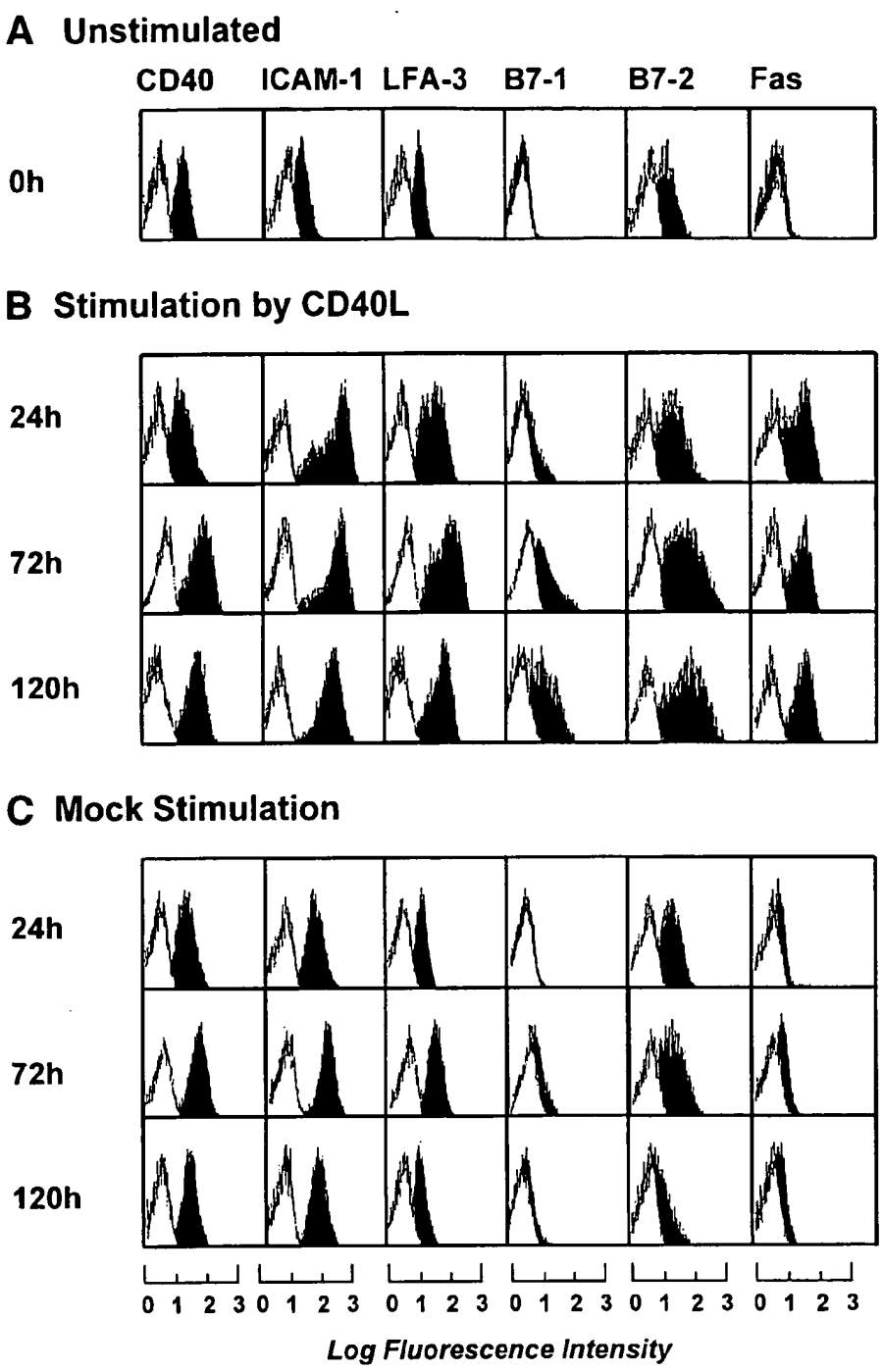


**Fig 3. Phenotypic characterization of unstimulated B-CLL cells and B-CLL cells stimulated in the presence of IL-4 (1 ng/mL) for 24, 72, and 120 hours by either CD40L-transfected or mock-transfected NIH3T3 fibroblasts. The results shown are from one experiment and are representative of three independent experiments.**

presence or absence of IL-2 (20 IU/mL) with γ-irradiated (75 Gy) native CLL and CD40-CLL cells for 72 hours. CD40-CLL cells were prepared by prestimulation with t-CD40L for 24, 72, and 120 hours. T-cell proliferation was assessed by incorporation of [$^3$H]thymidine added during the last 12 hours of the experiment. The highest stimulatory capacity of the CD40-CLL cells was achieved on day 3 of t-CD40L prestimulation (Fig 5). The experiments shown are representative for 3 different allogeneic T-cell donors and different cases of B-CLL examined.

In another set of experiments, we analyzed the proliferative response of highly purified allogeneic CD4$^+$ and CD8$^+$ T cells. For this purpose, we used γ-irradiated (75 Gy) native CLL and CD40-CLL cells (day 3) and incubated them for 72 hours with different ratios of highly (>95%) purified CD4$^+$ and CD8$^+$ T-cell subpopulations as well as unpurified peripheral blood

Table 3. Immunophenotype of B-CLL Cells Stimulated by CD40L

| | Recognition | | Adhesion | | Costimulation | | |
|---|---|---|---|---|---|---|---|
| Patient | MHC I | MHC II | ICAM-1 | LFA-3 | B7-1 | B7-2 | CD40 |
| CLL-1 | +++ | ++++ | ++ | + | ++ | ++++ | ++ |
| CLL-2 | ND | ++++ | ++ | ++ | ++ | +++ | ++ |
| CLL-3 | ND | +++ | ++ | + | + | +++ | +++ |
| CLL-4 | ND | ++++ | +++ | ++ | ++ | +++ | ++ |
| CLL-5 | +++ | ++++ | +++ | +++ | +++ | ++++ | +++ |
| CLL-6 | +++ | ++++ | ++ | +++ | ++ | +++ | +++ |
| CLL-7 | ND | +++ | ++ | ++ | ++ | +++ | ++ |
| CLL-8 | ++++ | ++++ | +++ | ++ | + | +++ | ++ |
| CLL-9 | +++ | ++++ | +++ | ++ | ++ | +++ | ++ |
| CLL-10 | ++++ | ++++ | +++ | ++ | + | +++ | ++ |
| CLL-11 | ++++ | ++++ | +++ | +++ | ++ | +++ | ++ |
| CLL-12 | ++++ | +++ | +++ | +++ | ++ | +++ | ++ |

Mean intensity of fluorescence: −, negative; +, >0.2-0.5 logarithm; ++, 0.5-1.5 logarithm; +++, 1.5-2.5 logarithm; ++++, >2.5 logarithm; ND, not done.

mononuclear cells (PBMCs). T-cell proliferation was assessed by [$^3$H]thymidine incorporation during the last 12 hours of the experiment. A representative experiment is shown in Fig 6. CD40-CLL cells but not native CLL cells induced a significant T-cell proliferation, regardless of whether unpurified PBMCs or CD4$^+$ or CD8$^+$ T cells were used. Moreover, CD4$^+$ T cells showed a significant stronger proliferative response than CD8$^+$ T cells. The strongest response was seen with PBMCs; this result is readily explained by the presence of some additional immune effector cells (eg, NK cells) or APCs (eg, dendritic cells) in the unpurified PBMC fraction.

*Different effector cells are induced by subsequent stimulation of allogeneic versus autologous T cells with CD40-CLL cells.* In the next step we determined whether CD40-CLL cells could be used to stimulate T-cell differentiation and expansion. For this purpose, we used highly purified allogeneic and autologous T cells and stimulated them weekly (days 0, 7, 14, and 28) with γ-irradiated (75 Gy) native and CD40-CLL cells at a ratio of 5:1. In both settings, it was possible to expand large numbers of

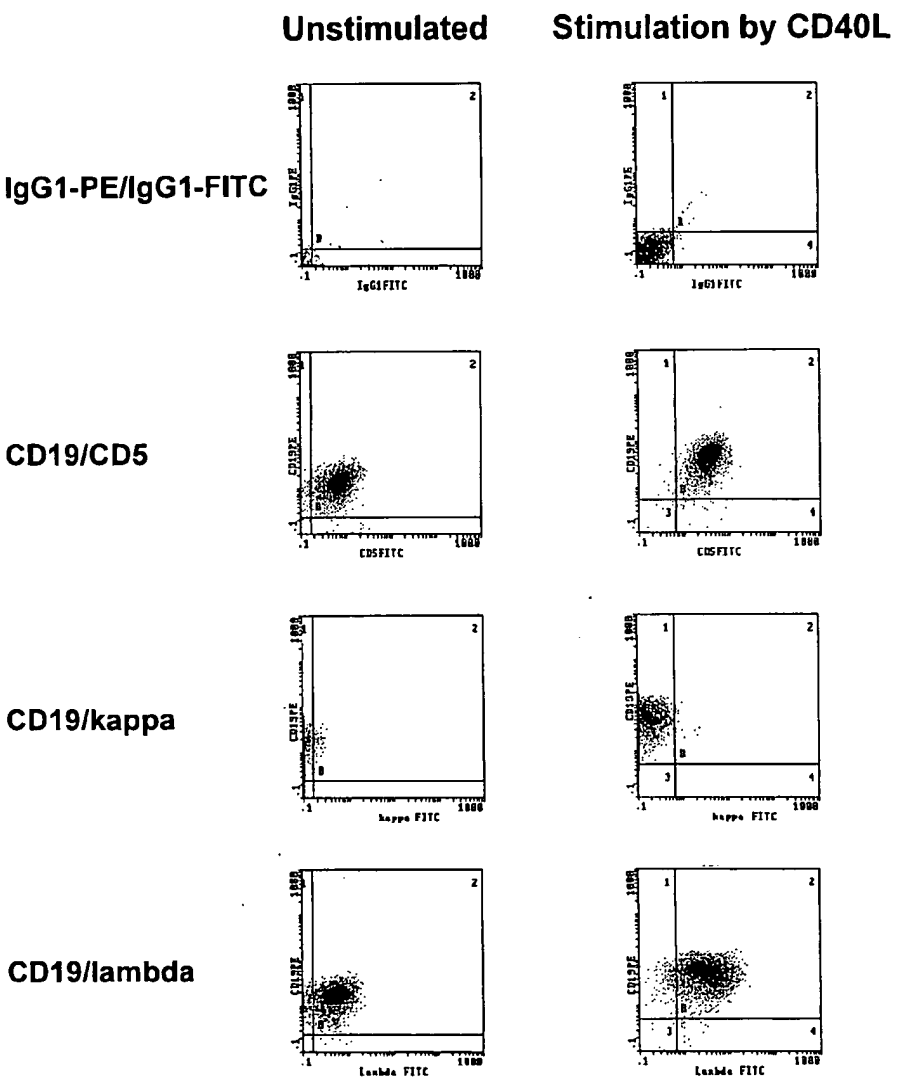


Fig 4. Representative immunophenotypic characterization of B-CLL cells before and after 3 days of CD40-stimulation in the presence of IL-4 (1 IU/mL) as determined by CD5/CD19 positivity and light chain restriction (patient no. 3). The results shown are from one experiment and are representative of three independent experiments.

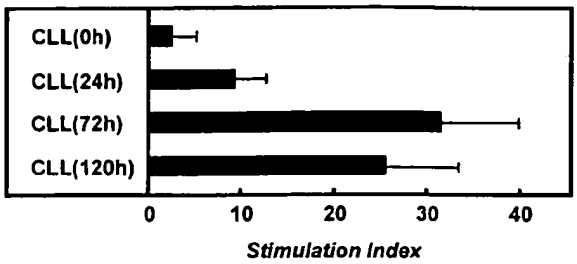


**Fig 5. Proliferative responses of purified allogeneic CD3+ T cells in the absence of IL-2 to γ-irradiated B-CLL cells (patient no. 12) either unstimulated or stimulated for 24, 72, and 120 hours by CD40L-transfected NIH3T3 fibroblasts. [³H] Thymidine incorporation was assessed for the last 12 hours of a 3-day culture. Appropriate controls (CD3+ T cells and B-CLL cells) were always less than 1,500 cpm. The stimulation index was calculated as $cpm_{(T\ cells\ +\ B\text{-}CLL\ cells)}/cpm_{(T\ cells)}$. Results are representative for three independent experiments and are expressed as the mean ± SD of the stimulation index.**

T cells in the presence of CD40-activated B-CLLs and exogenous IL-2 (20 IU/mL). In contrast, the expansion was not possible with native B-CLL cells, even in the presence of IL-2.

When T cells were monitored by flow cytometric analysis, we found that only CD40-activated B-CLLs were able to induce activation markers (CD25 and CD95). A marked difference was seen with regard to the CD4/CD8 ratio. In the allogeneic setting, consecutive stimulations with CD40-CLL cells caused a relative and absolute increase of $CD8^+$ T cells (up to 50% after 3 restimulations) in 4 of 5 cases investigated. In marked contrast, repetitive stimulations in the autologous setting caused an expansion of $CD4^+$ T cells (up to 90% after 3 restimulations) in 6 of 6 cases studied. No increase of $CD16^+/CD56^+$ NK cells was observed.

To further characterize the different effector functions of allogeneic versus autologous T cells, we performed 4-hour standard chromium release tests (see Materials and Methods). Native B-CLL cells, CD40-CLL cells, and NK-sensitive K562 cells were used as targets. These different effector cells were expanded until day 28 and then restimulated with CD40-CLL cells and native CLL cells in the presence of IL-2. A significant, cytolytic activity of T cells was only seen if allogeneic T cells from healthy donors were used (Fig 7B). At this time point, allogeneic T cells lysed both native B-CLL cells and CD40-CLL cells. No response against K562 cells was detected. Furthermore, no T-cell response was observed with T cells before stimulation with CD40-CLL (Fig 7A). In marked contrast, autologous T cells expanded by repetitive stimulation with CD40-CLL cells showed only a slight lytic activity against CD40-CLL cells and no lytic activity against native B-CLL cells or the NK-sensitive K562 cell line (Fig 7D). Taken together, the stimulation of allogeneic versus autologous T cells by CD40-CLL cells induced a fundamentally different response in that $CD8^+$ cells with cytolytic activity could only be expanded in the allogeneic system.

*CD40-CLL activated autologous CD4+ T cells show a Th1-type cytokine pattern.* In the next step we tried to characterize the autologous, $CD4^+$ T cells with respect to their cytokine release pattern. For this purpose, we restimulated $1 \times 10^5$ autologous T cells with $2 \times 10^4$ paraformaldehyde-fixed (1%) CD40-CLL cells. The supernatant was collected 48 hours later and analyzed for IL-4 and IFNγ. Figure 8 summarizes the data of 6 patients. In 4 of 6 patients tested, we detected predominantly IFNγ, suggesting a Th1-like immune response.

## DISCUSSION

This report investigates the potential of CD40L-stimulated CLL (CD40-CLL) cells to activate effector T cells for tumor vaccination. The essential finding of this report is that the quality of the autologous T-cell response against CD40-CLL cells differs dramatically from the allogeneic T-cell response against these cells. So far, it was known that CD40-ligation could be used in B-CLL cells to upregulate adhesion and costimulatory molecules.[25-27] Our study confirmed these findings by showing that stimulation of B-CLL cells by t-CD40L resulted in a significant upregulation of both adhesion and costimulatory molecules in B-CLL cells in a time-dependent

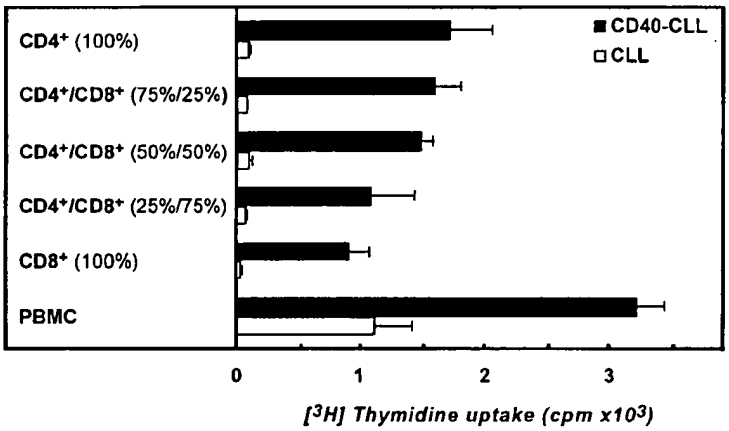


**Fig 6. Proliferative response of purified allogeneic $CD4^+$ and/or $CD8^+$ T cells as well as PBMCs not further purified to γ-irradiated native and CD40-stimulated B-CLL cells. Different CD4/CD8 ratios were tested. [³H] Thymidine incorporation was assessed for the last 12 hours of a 3-day culture and determined in cpm ± SD of triplicate determinations. The results shown are from one experiment and are representative for three independent experiments.**

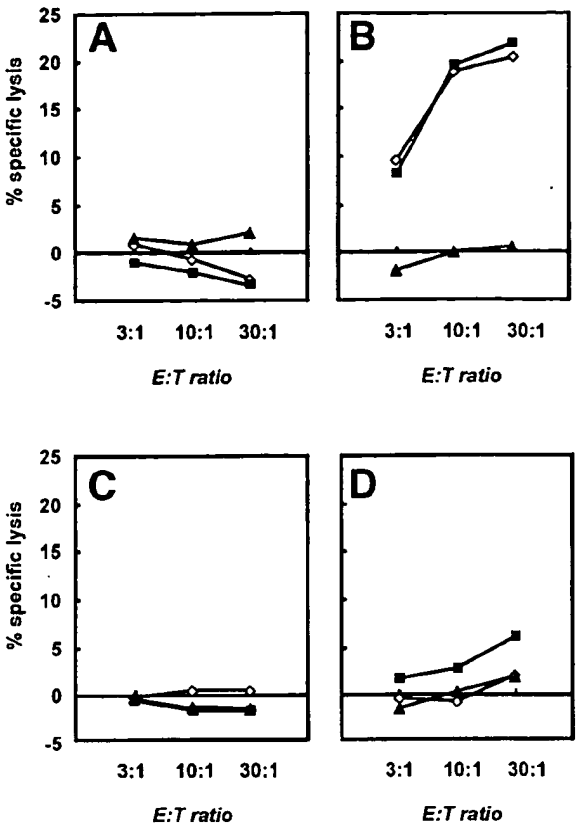


**Fig 7. Cytolytic response of unstimulated and activated allogeneic and autologous T cells as assessed in a standard 4-hour chromium release assay. A total of 2.5 × $10^3$ B-CLL cells (◇), CD40-CLL cells (■), and NK-sensitive K562 cells (▲) were placed in 96-well v-bottom plates and T cells were added at E:T ratios of 3:1, 10:1, and 30:1 in a final volume of 200 μL. Cytolytic response was expressed as the percentage specific lysis. (A) Cytolytic response of unstimulated T cells and (B) cytolytic response of T cells restimulated three times with CD40-CLL cells in an allogeneic setting. (C) Cytolytic response of unstimulated T cells and (D) cytolytic response of T cells restimulated three times with CD40-CLL cells in an autologous setting (patient no. 12). The results are representative for three independent experiments in the allogeneic system and three independent experiments in the autologous system.**

manner, regardless of previous treatment with cytostatic drugs. CD40-CLL cells were able to stimulate both the allogeneic and the autologous T-cell proliferation. The addition of IL-4 further enhanced the expression of both B7-1 and B7-2, whereas IFNγ or IL-2 reduced it compared with t-CD40L alone (Fig 2).

A high expression of B7-1 is critical for the induction of a CTL response.[28-30] To induce an effective immune response against B-CLL cells, consecutive T-cell stimulations were performed with CD40-CLL cells. Allogeneic CD40-CLL cells strongly stimulated both $CD4^+$ as well as $CD8^+$ T cells, as previously described.[27] In marked contrast, stimulation of autologous CD40-CLL cells strongly favored the outgrowth of $CD4^+$, but not $CD8^+$ T cells. With respect to the effector T-cell function, there were also marked differences. A cytolytic activity was only induced with allogeneic T cells stimulated by CD40-CLL cells. This resulted in a significant alloantigen-specific cytotoxic activity against both CD40-CLL and naive B-CLL cells, similar to previous findings.[27] In marked contrast, the stimulation of autologous T cells by CD40-CLL cells induced the expansion of a predominantly $CD4^+$, Th1-like effector cell population without cytolytic activity. Moreover and in contrast to findings in follicular lymphoma and acute lymphoblastic leukemia, CD40-CLL cells did not allow to expand autologous T cells with cytolytic activity.[31,32]

The inability of CD40-CLL cells to stimulate $CD8^+$ T cells in the autologous system can be explained by several alternative mechanisms. First, CD40 expressed on B-CLL cells may costimulate $CD4^+$ T cells rather than $CD8^+$ T cells.[33] Second, autologous peripheral blood T lymphocytes might be less efficient in mounting a cytolytic response against lymphoma cell antigens than T cells derived from the bone marrow or tumor-infiltrating lymphocytes.[32] Third, most if not all the anti-idiotype immune responses reported to date have been by $CD4^+$ cells (both Th1- and Th2-type).[34-36] Fourth, a distinct pattern of costimulatory molecules expressed on CD40-CLL cells may induce the preferential stimulation of Th1-like, $CD4^+$ T cells; for example, it is known that $CD8^+$ T cells require higher densities of B7-1 to attain an equivalent level of activation as $CD4^+$ T cells, and CD40-CLL cells may just not express enough B7-1 and/or B7-2 to activate $CD8^+$ T cells.[28] Sixth, the cytokine secretion by stimulated T cells themselves may contribute to the modulation of costimulatory molecules on CD40-CLL cells. These different mechanisms act probably in concert in regulating the threshold by which an ongoing T-cell response is maintained.

However, the exact factors preventing the outgrowth of autologous $CD8^+$ effector T cells with antileukemic activity remain to be elucidated. It seems highly unlikely that autologous, peripheral blood T lymphocytes are intrinsically incapable of mounting a CTL response in B-CLL patients, because the generation of tumor cell-specific CTLs from the peripheral blood of B-CLL patients has been recently demonstrated.[37]

It will be of interest to learn whether the $CD4^+$ Th1 cells stimulated by CD40-CLL cells are able to induce B-CLL cell death, eg, by triggering a Fas-dependent apoptotic pathway, as shown in human tonsillar and Burkitt's lymphoma B cells.[38-40] Preliminary experiments in our laboratory showed that CLL cells were indeed rapidly eliminated in coculture with these $CD4^+$ T cells (R. Buhmann, unpublished data).

The CD40-CD40L interaction is crucial for the immune response. The ability to trigger and to regulate the induction and expression of CD40L on $CD4^+$ cells appears to be of paramount importance to the generation of the T-cell–dependent antigen response.[41,42] An excess of CD40-expressing leukemia cells might interfere with these cognate T-cell responses and provoke an aquired CD40L deficiency syndrome in B-CLL.[43] Previous studies and our data suggest that these defects can be restored at least in part by an effective T-cell activation, with lymphoma or leukemia cells expressing costimulatory molecules at sufficient density.[6,7,31] Activation of naive T cells can be brought by any APC, as long as sufficient levels of one or more accessory molecules are expressed. Later, during the process of T-cell activation and differentiation, the requirements for full T-cell stimulation decrease, as T cells become more responsive to a

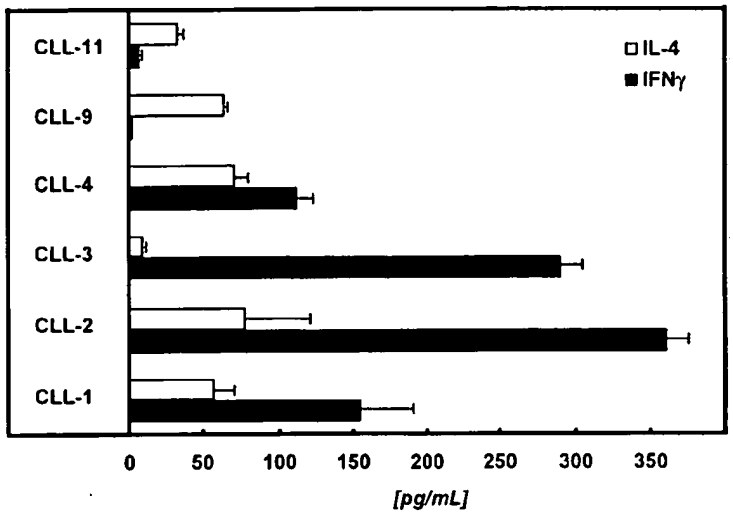


**Fig 8. Autologous T cells of 6 different patients were challenged with CD40-CLL cells at an E:T ratio of 5:1. Supernatants were collected 48 hours later and IL-4 and IFNγ were measured by ELISA (detection limit of the IL-4 assay is <5 pg/mL; detection limit of the IFNγ assay is <5 pg/mL).**

particular antigen.[42,44,45] Thus, an APC that is only weakly stimulatory for naive T cells can become an efficient stimulator for preactivated T cells. Accordingly, native B-CLL cells might have properties of weakly stimulating APCs that need the concomitant presence of strong APCs (such as CD40-CLL cells or dendritic cells) to fully activate T cells.

Taken together, our results suggest that CD40 activation of B-CLL cells may reverse T-cell anergy against the neoplastic clone. The results might provide new perspectives for the immunotherapy of B-CLL, similar to previous observations in follicular lymphoma[7,31] and pre-B acute lymphoblastic leukemia (ALL).[6] Most importantly, the clinical application to produce tumor vaccines or to generate stimulator cells for adoptive immune transfer strategies in B-CLL by this approach will meet less practical limitations than in other lymphoid malignancies, because B-CLL cells are readily obtained from peripheral blood. Moreover, the identity of CD40-CLL cells can be rapidly tested by measuring κ or λ light chain restriction and CD5/CD19 coexpression on the tumor cell surface by flow cytometry. This will facilitate the practical implementation of these approaches in the adjuvant therapy of B-CLL.

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